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Alergia a alimentos: reactividad alergénica y efectos de los tratamientos enzimáticos, térmicos y de presión

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Beatriz Cabanillas Martín

Director

Jesús Fernández Crespo

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ALERGIA A ALIMENTOS: REACTIVIDAD ALERGÉNICA Y EFECTOS DE LOS TRATAMIENTOS ENZIMÁTICOS, TÉRMICOS Y DE PRESIÓN

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INTRODUCCIÓN

Alergia a alimentos

Definición, prevalencia, gravedad y relevancia socio-sanitaria.

La alergia alimentaria se define como una reacción clínica adversa y reproducible debida a una respuesta del sistema inmune tras la exposición a un alimento determinado. La alergia alimentaria puede estar mediada por inmunoglobulinas E (IgE) o por otros mecanismos inmunológicos. Por otro lado, las reacciones adversas reproducibles que no están mediadas por mecanismos inmunológicos se denominan intolerancias alimentarias. El diagnóstico de la alergia a un alimento mediada por IgE requiere: que el paciente esté sensibilizado al mismo, es decir, la presencia en su organismo de anticuerpos IgE específicos a uno o más de sus alergenos y que se produzcan síntomas clínicos tras la exposición a ese alimento (Burks et al., 2011).

La prevalencia de la alergia alimentaria es difícil de establecer, en gran parte por las diferencias en la metodología de los distintos estudios epidemiológicos llevados a cabo. En lo que se refiere a los alimentos considerados más frecuentes, sin embargo, tanto la incidencia como la prevalencia de la alergia alimentaria pueden haber cambiado a lo largo del tiempo; además el diseño de los estudios de evaluación de la alergia a alimentos no es uniforme. En un meta-análisis llevado a cabo por Rona et al (2007), la prevalencia total de la alergia a leche de vaca, cacahuete, pescado o crustáceos era de un 12% en niños y un 13% en adultos cuando la evaluación de la alergia alimentaria se realizó atendiendo únicamente a los síntomas referidos por los pacientes. Sin embargo, cuando se analizaron tanto los síntomas referidos como la sensibilización y las pruebas orales doble ciego controlado con placebo llevadas a cabo para estos alimentos el porcentaje se redujo al 3% para adultos y niños.

La gravedad de las reacciones alérgicas a alimentos es multifactorial y variable, no pudiéndose predecir de forma precisa por el cuadro clínico presentado por el

paciente en reacciones anteriores; tampoco por los niveles de anticuerpos IgE específicos ni por los resultados de las pruebas cutáneas (Boyce et al., 2010). El factor probablemente más predictivo de las reacciones graves es la coexistencia de asma en el paciente, especialmente en reacciones alérgicas a alimentos como cacahuete y otros frutos secos.

En la actualidad, la alergia alimentaria es la principal causa de reacciones anafilácticas tratadas en los servicios de urgencia hospitalarios en los países occidentales. En un estudio realizado en Estados Unidos se ha estimado que la alergia a alimentos suma 125.000 visitas/año a los servicios de Urgencias, de las cuales 14.000 corresponden a reacciones anafilácticas (Ross et al., 2008). El cacahuete y otros frutos secos son los causantes de la mayoría de las anafilaxias mortales en el mencionado país (Bock et al., 2001; Bock et al., 2007). La alergia alimentaria tiene un impacto económico importante en la sociedad y en el sistema sanitario; además, numerosos estudios en los últimos 10 años han demostrado que ésta está asociada con un impacto negativo en la calidad de vida de los pacientes, con efectos a nivel físico, social y emocional o psicológico (Lieberman y Sicherer, 2011). Algunas alergias específicas, como la producida por cacahuete, tienen un impacto sobre la calidad de vida y las relaciones familiares comparable a algunas enfermedades reumatológicas (Primeau et al., 2000).

Diagnóstico de la alergia alimentaria mediada por IgE

Aunque la historia clínica y el examen físico constituyen la base del proceso de evaluación de la alergia alimentaria, éstos no son suficientes para el diagnóstico; se deben realizar otros estudios complementarios: las pruebas cutáneas a los alimentos referidos por el paciente y la determinación de IgE sérica a los mismos se emplean para

detectar un mecanismo de hipersensibilidad inmediata mediado por anticuerpos IgE a un determinado alimento, sin embargo no establecen el diagnóstico de la alergia clínica. Con la excepción de reacciones graves referidas a un alimento, la verificación de la reactividad clínica requiere de provocaciones orales o pruebas de tolerancia al mismo correctamente diseñadas (Burks et al., 2011). Estas pruebas consisten en la administración gradual por vía oral de un determinado alimento bajo supervisión médica para determinar tolerancia o reactividad clínica. Éste es el único método objetivo de diagnóstico de alergia a alimentos y requiere de la información obtenida en la historia clínica para su correcto diseño en cuanto al alimento con el que realizar la provocación, cantidad mínima del mismo, tiempo transcurrido entre ingestión y aparición de los síntomas de la reacción referida, etc (Bock et al., 1988). La provocación oral con alimentos puede ser: abierta, en la que el paciente y personal sanitario identifican el alimento; simple ciego controlado con placebo, en la que se enmascara el alimento para que no sea identificado por el paciente ni lo distinga del placebo y doble ciego controlado con placebo, en la que ni el paciente ni el personal sanitario que realiza esta técnica identifican alimento o placebo durante la realización de la misma. Este método constituye la única prueba de tolerancia objetiva debido a que elimina el sesgo del paciente y del personal sanitario (Boyce et al., 2010; Mari y Scala, 2006; Sicherer y Sampson, 2010).

Tratamiento

Una vez que el diagnóstico de alergia a un alimento ha sido establecido se debe adoptar una eliminación estricta del mismo de la dieta del paciente. Sin embargo, la ingestión accidental del alimento al que el paciente es alérgico puede ocurrir incluso tomando las medidas correctas de evitación, en situaciones como comidas fuera del domicilio o

ingestión de alimentos sin etiquetado. La administración de adrenalina constituye el primer tratamiento de las reacciones alérgicas agudas y sistémicas (Simon FE, 2009); los antihistamínicos se emplean como tratamiento de los síntomas de reacciones alérgicas leves (Boyce et al., 2010).

Las investigaciones sobre nuevas opciones terapéuticas para la alergia alimentaria se han intensificado en los últimos años. La inmunoterapia oral o sublingual específica de alergeno se ha empleado para inducir desensibilización clínica a alimentos, pero esta terapia puede acarrear el riesgo de producir reacciones graves y actualmente no se recomienda su uso rutinario en la práctica clínica. La inmunoterapia con alergenos que presentan reactividad cruzada no se recomienda para el tratamiento de alergia alimentaria mediada por IgE (Burks et al., 2011).

Alergenos alimentarios y reactividad cruzada

Los alergenos alimentarios son proteínas o glicoproteínas que inducen la producción de anticuerpos IgE y que, tras la unión con éstos, desencadenan la liberación de mediadores inflamatorios por las células diana (Lehrer et al., 1997). Los alergenos alimentarios pueden clasificarse, en función de la vía de sensibilización, en: alergenos de clase I, cuando la sensibilización tiene lugar a través del tracto gastrointestinal tras la ingestión del alimento y de clase II, en el caso de que ésta se produzca por inhalación y las proteínas alergénicas presenten reactividad cruzada con alergenos alimentarios. La mayoría de los alergenos de clase I son glicoproteínas que tienen un peso molecular entre 10 y 70 kDa. Generalmente son resistentes al calor, al pH ácido y a la digestión enzimática. Los alergenos que sensibilizan por inhalación suelen ser proteínas termolábiles y susceptibles de alteración y pérdida de antigenicidad por proteolisis enzimática o pH ácido (Sampson y Burks, 2008).

La mayoría de los alergenos alimentarios vegetales pueden ser agrupados en unas pocas familias y superfamilias de proteínas, altamente conservadas y ubicuas. Numerosos alergenos pertenecen a la superfamilia de las cupinas (como las globulinas 7S y 11S) o de las prolaminas (albúminas 2S, proteínas de transferencia de lípidos, α-amilasas/inhibidores de la tripsina, etc). Gran parte de los alergenos vegetales son homólogos a las proteínas de defensa o PR (*Pathogenesis Related*), constituidas por una colección heterogénea de 14 familias de proteínas cuya función principal es la defensa de las plantas frente a agresiones externas ambientales o biológicas (Breiteneder y Radauer, 2004).

Los alergenos alimentarios de origen animal de mayor relevancia están presentes en leche, huevo, crustáceos, moluscos y pescados. Las proteínas alergénicas de la leche de mamífero pertenecen mayoritariamente a 3 familias: α-lactoalbúminas, β-lactoglobulinas y caseínas. El ovomucoide y la ovoalbúmina son los alergenos más importantes del huevo y se encuentran presentes en la clara. En el caso de crustáceos y moluscos, las tropomiosinas son proteínas que juegan un papel biológico clave en la regulación de la contracción muscular y son alergenos importantes. Las parvalbúminas, por su parte, tienen como función biológica la relajación de las fibras musculares y son los alergenos mayoritarios de pescado (Chapman et al., 2007).

Alergenos de leguminosas

Las leguminosas son plantas dicotiledóneas que pertenecen al orden botánico Fabales. La familia *Papilionaceae* es la familia más numerosa y a ella pertenecen las especies responsables de la mayoría de las reacciones alérgicas por leguminosas: *Arachis hipogaea* (cacahuete), *Glycine max* (soja), *Lens culinaris* (lenteja), *Cicer arietinum* (garbanzo), *Pisum sativum* (guisante), *Lupinus albus* (lupino), etc.

Las proteínas alergénicas de leguminosas identificadas hasta el momento pertenecen a grupos diversos. Dentro de la superfamilia de las cupinas, las globulinas son proteínas de almacenamiento de semillas que constituyen un grupo de alergenos importantes en leguminosas (Breiteneder y Radauer, 2004). Se clasifican en: globulinas 7S o vicilinas y globulinas 11S o leguminas. Las globulinas 7S maduras están compuestas por trímeros de 150 a 190 kDa, presentando sus subunidades pesos moleculares entre 40 y 80 kDa. El alergeno mayor de cacahuete, Ara h 1, es una de la vicilinas mejor caracterizadas. La estructura trimérica de Ara h 1 es altamente estable y sus epítopos IgE se encuentran localizados en la región de contacto entre subunidades lo que les protege de la digestión por proteasas (Maleki et al., 2000a). Sin embargo se ha demostrado que sus subunidades independientes son rápidamente digeridas por pepsina (Koppelman et al., 2010). La vicilina de lenteja, Len c 1, ha sido identificada como uno de sus alergenos mayores (López-Torrejón et al., 2003). Tiene un peso molecular de 48 kDa y recientemente se han caracterizado sus epítopos IgE, que se localizan en el extremo C-terminal estando expuestos en la superficie de la proteína (Vereda et al., 2010). Las globulinas 11S maduras, por su parte, son proteínas hexaméricas. Cada subunidad está constituida por un polipéptido de entre 30-40 kDa, unido mediante un puente disulfuro a un polipéptido de aproximadamente 20 kDa (Mills et al., 2002). Ara h 3 es una globulina 11S de cacahuete que ha sido descrita como alergeno. Los epítopos IgE de Ara h 3 se han identificado principalmente en la subunidad mayor de la proteína (Rabjohn et al., 1999), aunque la subunidad menor se ha descrito como un alergeno mayor en un grupo de niños alérgicos a cacahuete en Italia (Restani et al., 2005).

La superfamilia de las prolaminas agrupa a varios alergenos de leguminosas. La mayoría de estas proteínas se caracteriza por tener un bajo peso molecular, ser ricas en cisteínas y poseer una estructura tridimensional con un elevado porcentaje de α-hélices.

La superfamilia incluye importantes familias de alergenos vegetales como las albúminas 2S y las proteínas de transferencia de lípidos (LTPs), entre otras (Breiteneder y Radauer, 2004). Las albúminas 2S son proteínas de almacenamiento de semillas, típicamente heterodiméricas de aproximadamente 8-10 y 3-4 kDa que se unen mediante cuatro puentes disulfuro. La estructura proteica altamente conservada y compacta juega un papel crucial en la resistencia de estas proteínas (Moreno y Clemente, 2008). Ara h 2, Ara h 6 y Ara h 7 son los alergenos que pertenecen a la familia de las albúminas 2S en cacahuete. Se ha comprobado que Ara h 2 actúa como un inhibidor de la tripsina y que protege al alergeno Ara h 1 de la degradación proteica por esta enzima (Maleki et al., 2003). Las LTPs son moléculas monoméricas de 7 a 9 kDa que presentan cuatro puentes disulfuro en su estructura. Las LTPs se acumulan normalmente en la capa epidérmica de los órganos vegetales (van Ree, 2002). Las LTPs también son resistentes a la proteólisis o cambios de pH y tienen una amplia distribución entre frutas, frutos secos, semillas y vegetales.

Las proteínas de defensa son una colección de familias proteicas no relacionadas entre sí que se sintetizan en respuesta a agresiones tales como ataques de patógenos, heridas, sustancias químicas como fitohormonas, metales, rayos ultravioleta y condiciones de crecimiento desfavorables y funcionan por tanto como defensa de las plantas. Al menos una veintena de estas proteínas han sido descritas como alergenos, así, entre los 14 grupos de proteínas de defensa, los grupos 2, 3, 4, 5, 8, 10 y 14 contienen alergenos (Midoro-Horiuti et al., 2001). Los miembros de la familia de proteínas de defensa tipo PR-10 presentan una elevada similitud de secuencia con el alergeno principal de abedul, Bet v 1, uno de los alergenos vegetales más conocidos. Son proteínas de alrededor de 18 kDa que se han descrito como alergenos en numerosas especies, entre ellas cacahuete (Ara h 8) y soja (Gly m 4). Estos alergenos están

relacionados con el Síndrome Alérgico Oral (SAO) (Rodríguez, 2004). Los alergenos de leguminosas se resumen en la Tabla 1.

Leguminosa	**Alergeno**	**Nombre bioquímico**	**Peso molecular (kDa)**
Cacahuete (*Arachis hypogaea*)	Ara h 1	Globulina 7S	64
	Ara h 2	Albúmina 2S	17
	Ara h 3	Globulina 11S	60
	Ara h 4	Globulina 11S	37
	Ara h 5	Profilina	15
	Ara h 6	Albúmina 2S	15
	Ara h 7	Albúmina 2S	15
	Ara h 8	PR-10	17
	Ara h 9	LTP	10
	Ara h 10	Oleosina	16
	Ara h 11	Oleosina	14
Lenteja (*Lens culinaris*)	Len c 1	Globulina 7S	47
	Len c 2	Proteína biotinilada específica de semilla	66
	Len c 3	LTP	9
Soja (*Glycine max*)	Gly m 1	Proteína hidrofóbica	7
	Gly m 2	Defensina	8
	Gly m 3	Profilina	14
	Gly m 4	PR-10	17
	Gly m 5	Globulina 7S	
	Gly m 6	Globulina 11S	
Guisante (*Pisum sativum*)	Pis s 1	Globulina 7S	44
	Pis s 2	Globulina 7S	63

Tabla 1: Alergenos de leguminosas

Reactividad cruzada

El fenómeno de la reactividad cruzada en alergia ocurre cuando los anticuerpos IgE originalmente específicos de un determinado alergeno reconocen una proteína similar de otra fuente. La interacción con tales proteínas homólogas puede desencadenar una reacción alérgica o bien puede ser completamente irrelevante a nivel clínico (Ferreira et al., 2004). En algunos casos, la reactividad cruzada a nivel de anticuerpos IgE se basa en secuencias aminoacídicas altamente homólogas. En general, para que exista reactividad cruzada tiene que existir más de un 70% de homología en la secuencia. Las proteínas con menos del 50% tienen menos probabilidades de presentar reactividad cruzada (Aalberse, 2000). En otros casos la reactividad cruzada se basa en una homología en la estructura tridimensional de las proteínas (Bonds et al., 2008).

Como se ha mencionado anteriormente, la mayoría de los alergenos de leguminosas pueden ser agrupados en unas pocas familias de proteínas, altamente conservadas y ubicuas. Éstas presentan un alto grado de homología entre distintas especies, lo que explicaría la gran extensión de sensibilizaciones a alimentos de la misma familia y en otros casos a otros no relacionadas taxonómicamente. Se ha demostrado que la sensibilización a nivel de anticuerpos IgE a múltiples leguminosas es habitual en pacientes clínicamente alérgicos a alguna de ellas. Bernhisel-Broadbent et al (1989a) estudiaron 62 niños con alergia al menos a una leguminosa y encontraron que el 79% presentaban anticuerpos IgE específicos en suero a más de una especie, y el 37% a las seis estudiadas (cacahuete, soja, guisante, garbanzo, judía y haba). Sin embargo, a pesar de la elevada reactividad cruzada inmunológica, se ha demostrado que la reactividad cruzada clínica no es tan común en esta familia. Bernhisel-Broadbent y Sampson (1989b) realizaron pruebas orales doble ciego controlado con placebo con leguminosas en 69 niños atópicos, con pruebas cutáneas positivas al menos a una de

ellas. Se confirmaron 43 reacciones en 41 pacientes. Sólo 2 de los 41 pacientes reaccionaron a más de una leguminosa. La reactividad cruzada clínica en la familia *Leguminosae* podría ser más elevada cuando se tienen en cuenta otros miembros de esta familia. Pascual et al (1999) demostraron que de 20 niños con alergia clínica a lenteja, 6 presentaban alergia a garbanzo, 2 a guisante y uno a judía verde.

La alergia a lupino (*Lupinus albus*) fue descrita por primera vez en 1994 en una paciente con alergia clínica a cacahuete. La reacción se debió a la ingestión de pasta enriquecida con harina de lupino (Hefle et al., 1994). Desde entonces la alergia a lupino ha sido documentada principalmente en pacientes con alergia a cacahuete, aunque también se han descrito casos de alergia clínica exclusivamente a lupino (Wassenberg et al., 2007). En Francia, Moneret-Vautrin et al. (1999) encontraron que de 6 niños con alergia clínica a cacahuete, 5 presentaban provocación doble ciego controlado con placebo con resultado positivo con lupino. La harina de esta leguminosa se utiliza actualmente en numerosos alimentos, principalmente como suplemento de la harina de trigo debido a sus propiedades nutricionales (Guillamón et al., 2010a), esto hace que se pueda encontrar en numerosos alimentos, en ocasiones en pequeñas cantidades como alergeno oculto, lo que puede dar lugar a reacciones alérgicas (Faeste et al., 2004; Sanz et al., 1995). La reactividad cruzada clínica entre lupino y otras leguminosas distintas a cacahuete no ha sido estudiada hasta la fecha.

Otro caso de reactividad cruzada se produce en el denominado “síndrome látex-frutas”. La existencia de este síndrome fue propuesta en 1994 en base a la observación clínica de una elevada tasa de hipersensibilidad inmediata a frutas en un grupo de 25 pacientes con alergia a látex. Las frutas implicadas fueron principalmente castaña, aguacate y plátano, aunque también se constató alergia a kiwi, papaya y otros alimentos (Blanco et al., 1994). La reactividad cruzada entre látex y varias frutas se ha demostrado

mediante inhibición de *radio allergosorbent test* (RAST), además se han identificado numerosos antígenos comunes mediante experimentos de inhibición de inmunoblot (Blanco, 2003). En una serie de 136 pacientes con alergia a látex, el 42,6% de los mismos refirieron reacciones graves tras la ingestión de un amplia variedad de frutas, con el kiwi y el plátano como los más comunes. Mediante experimentos de inhibición de RAST se demostró reactividad cruzada entre látex y aguacate, plátano, castaña, kiwi, papaya, maracuyá, higo, melón, mango, piña, melocotón y tomate (Brehler et al., 1997). Algunos de los alergenos implicados en la reactividad cruzada en el síndrome látex-frutas han sido caracterizados. La proheveína o Heb b 6.01 de látex, es una proteína de unión a quitina con un peso molecular de aproximadamente 20 kDa. La capacidad de unir IgE se atribuye principalmente a su dominio N-terminal, conocido como heveína o Hev b 6.02 con un peso molecular de 4,7 kDa (Alenius et al., 1996). Por su lado, las quitinasas de clase I de castaña y aguacate incluyen un dominio N-terminal similar a heveína en su secuencia lo que podría explicar la reactividad cruzada látex-frutas. Estas quitinasas han sido clonadas y expresadas y se ha documentado la reactividad cruzada con heveína (Chen et al., 1998; Sowka et al., 1998).

Evaluación de la alergenicidad de alimentos

Uno de los primeros pasos para la evaluación de la alergenicidad de alimentos debe ser la identificación de un grupo de estudio de pacientes alérgicos con una buena caracterización clínica basada en una provocación oral doble ciego controlada con placebo positiva o bien una historia de anafilaxia documentada con el alimento específico, en cuyo caso queda excluida la realización de una provocación oral (Lucas y Atkinson, 2008). El suero y ciertas células del sistema inmune de estos pacientes serán esenciales en la evaluación de la alergenicidad de los alimentos así como en la

identificación de alergenos específicos. Para llevar a cabo la identificación de un alergeno es necesario analizar una serie de características responsables de su alergenicidad (resistencia a la digestión y procesado, relación con alergenos conocidos, etc). Sin embargo, estas características no son exclusivas de las proteínas alergénicas y por tanto no las identifica por completo. Así, no existen reglas para predecir la alergenicidad potencial de las proteínas de un alimento, sólo una combinación de diferentes métodos *in vivo*, *in vitro* e *in silico* con los que se pueden evaluar las características alergénicas de un alimento determinado (Sanchez-Monge y Salcedo, 2005).

Las proteínas alergénicas pueden ser analizadas si se separan físicamente mediante SDS-PAGE y se realiza un inmunoblot que incluye transferencia de las proteínas a membrana y posterior incubación con suero de pacientes alérgicos (Sanchez-Monge y Salcedo, 2005). En el informe FAO / OMS de expertos sobre la alergenicidad de alimentos modificados por medios biotecnológicos se desaconseja la utilización de una combinación de muchos sueros (en número superior a 5) para la evaluación de la alergenicidad de un alimento, ya que los anticuerpos específicos podrían quedar diluidos en dicho *pool*. El informe recomienda la utilización de una selección de 25 muestras individuales de suero con niveles elevados de IgE que reaccionen con el alimento específico (FAO/WHO, 2001). En un inmunoblot las bandas individuales típicamente representan una sola proteína, sin embargo, si varias proteínas poseen el mismo peso molecular, éstas migrarán de forma similar y formarán una sola banda (Poms et al., 2004). Este problema se puede resolver empleando la electroforesis bidimensional, que permite la separación de muestras con proteínas heterogéneas en base a sus puntos isoeléctricos y pesos moleculares (Larbi y Jefferies, 2009). Actualmente, la técnica de ELISA es el método más utilizado en los laboratorios de la industria alimentaria para

detectar y cuantificar alergenos ocultos en alimentos (Poms et al., 2004), también es el método de elección cuando se evalúa la alergenicidad de hidrolizados proteicos debido a la sensibilidad y a la relativa simplicidad de la técnica (Clemente et al., 1999b).

Otro sistema basado en el denominado enzimoinmunoensayo con marcaje fluorimétrico (FEIA, por sus siglas en inglés), es el método más utilizado en la práctica clínica para la determinación de IgE específica en suero. Para ello se emplea una curva dosis-respuesta de IgE total para interpolar los datos de IgE específica. Las unidades empleadas son kilo unidades de IgE por litro (kU/L: 1 U = 2,4 ng de IgE) (Sanchez-Monge y Salcedo, 2005). Aunque esta técnica se emplea principalmente en la práctica clínica, los ensayos de inhibición de CAP (nombre comercial de un tipo de FEIA) se pueden emplear en experimentos de competición de la IgE específica para evaluar la reactividad cruzada entre alergenos. Estos ensayos se basan en que antígenos en solución pueden unir IgE específica inhibiendo así la unión de IgE al antígeno inmovilizado en la fase sólida (Poms et al., 2004).

La confirmación de la alergenicidad de una proteína se puede determinar mediante pruebas cutáneas, ensayos de liberación de histamina o cuantificación de marcadores del proceso de desgranulación (Lucas y Atkinson, 2008). Estos métodos evalúan la capacidad de una proteína de activar células efectoras como basófilos o mastocitos. Los ensayos de liberación de histamina se realizan incubando sangre heparinizada con concentraciones crecientes del supuesto alergeno y cuantificando, después de la centrifugación de las células, la histamina mediante técnicas como ELISA, fluorimetría, etc. En el caso de la cuantificación de marcadores de desgranulación se utilizan dos proteínas de membrana que se encuentran en los basófilos activados, CD63 y CD203c. Estos marcadores, se cuantifican mediante citometría de flujo empleando anticuerpos monoclonales conjugados con fluorocromos.

CD203c se expresa únicamente en basófilos y mastocitos, mientras que CD63 se expresa también en otras células como monocitos, teniendo que utilizar un segundo reactivo específico de basófilo en la prueba (Sanchez-Monge y Salcedo, 2005).

Procesos de modificación de alergenicidad de alimentos: naturales, procesamientos tecnológicos, genéticos, etc.

Naturales

Muchos de los alergenos derivados de plantas pertenecen al grupo de proteínas de defensa, las cuales muestran un perfil de expresión variable como respuesta a factores endógenos o exógenos, mientras que la expresión de otros alergenos depende de los procesos de maduración y de las diferentes variedades de cultivo (Constantin et al., 2009). En trigo se ha demostrado una variación en la expresión de alergenos durante la maduración; algunos se expresan mayoritariamente en los estadios iniciales y otros lo hacen en los estadios tardíos (Constantin et al., 2009). Chung et al (2003) demostraron que el grado de maduración de la semilla de cacahuete podría estar relacionado con un aumento de la alergenicidad. Además, el proceso de tostado incrementaría dicha alergenicidad. En experimentos realizados con los alergenos de cacahuete Ara h 1, Ara h 2 y Ara h 3 -proteínas de almacenamiento de semillas- se halló que los transcritos de estos alergenos presentes en todos los estadios de maduración de las semillas fueron indetectables durante la germinación y en estadios posteriores. Por tanto la transcripción de estos alergenos no ocurre durante la germinación y las proteínas Ara h 1, Ara h 2 y Ara h 3 almacenadas son hidrolizadas en las primeras horas de germinación para la obtención de aminoácidos para la síntesis proteica en la planta en crecimiento (Kang et al., 2007). En cuanto a los alergenos de trigo, se han demostrado diferencias entre distintas variedades de cultivo, lo cual podría abrir vías para el cultivo de variedades

con alergenicidad reducida (Constantin et al., 2009). En manzana, sobre la base de estudios previos realizados en pacientes con SAO a esta fruta, la variedad Santana ha sido comercializada en los Países Bajos desde 2006 como un cultivo con reducidas propiedades alergénicas (Vlieg-Boerstra et al., 2011). Así mismo, se han realizado estudios para identificar variedades de cultivo de cacahuete con una menor concentración de ciertos alergenos. Schmidt et al (2009) demostraron que las variedades de cacahuete de Indonesia contenían niveles inferiores del alergeno mayor de cacahuete, Ara h 1. Sin embargo, en estudios posteriores mediante experimentos de liberación de mediadores, se ha demostrado que la alergenicidad del extracto completo de dicha variedad no difiere de la del extracto de cacahuete estándar (Krause et al., 2010).

Procesamiento de alimentos

El procesamiento de alimentos, un medio imprescindible para su conservación, modificación de las características organolépticas, textura, así como para la eliminación de toxinas o de factores antinutricionales, tiene distinto impacto en las propiedades alergénicas de los alimentos dependiendo del método empleado (Mills y Mackie, 2008). El procesamiento de alimentos puede incluir: secado, ultrafiltración, osmosis inversa, irradiación gamma, calentamiento por infrarrojos, procesamiento a altas presiones, sonicación, evaporación, esterilización, tostado, horneado, congelado, etc (Sathe y Sharma, 2009). El impacto que tiene el procesamiento en las propiedades alergénicas de los alimentos es variado debido a la complejidad inherente a los mismos. Algunas de las de modificaciones que podrían tener lugar en las proteínas alimentarias durante el procesamiento incluirían efectos tales como desnaturalización, agregación y modificaciones químicas (Mills et al., 2009). Éstas pueden dar lugar al aumento, la disminución o simplemente no afectar a la alergenicidad de los alimentos (Sathe y

Sharma, 2009). Para entender cómo el procesamiento específico de alimentos puede afectar a los alergenos alimentarios, es importante apreciar no sólo la diversidad de los métodos empleados sino también la naturaleza molecular de los alergenos alimentarios y sus epítopos. Un alergeno puede presentar dos tipos de epítopos: lineales y conformacionales. En el primer caso, los aminoácidos que determinan si un alergeno se unirá o no a IgE son conocidos como aminoácidos críticos. Cualquier modificación, deleción o substitución de éstos podría resultar en la reducción o eliminación de la capacidad de ser reconocido por IgE. Si se trata de un epítopo conformacional, los cambios en dicha conformación podrían dar lugar a una modulación de su capacidad alergénica. El procesamiento de alimentos, bajo unas condiciones apropiadas, puede alterar la naturaleza de los epítopos. Por ejemplo, los epítopos conformacionales podrían ser modificados como resultado de un tratamiento que produzca una desnaturalización proteica. La hidrólisis ácida o enzimática de una proteína alergénica podría producir la disrupción de epítopos en un alergeno determinado. Sin embargo hay que tener en cuenta que el procesamiento, dependiendo del alergeno y del método que se emplee en el mismo, podría no afectar a las propiedades de determinados alergenos (Sathe y Sharma, 2009).

Durante el procesamiento de los alimentos pueden tener lugar distintas modificaciones químicas. Una de ellas es la reacción entre grupos amino libres de proteínas y grupos aldehído de azúcares reductores, en lo que se conoce como reacción de Maillard. Esta glicosilación no enzimática da lugar a bases de Schiff, las cuales pueden reorganizarse intramolecularmente y alcanzar un equilibrio más estable, aunque reversible, denominándose productos de Amadori. Estos productos a su vez pueden sufrir reacciones espontáneas intra e intermoleculares, deshidrataciones y condensaciones para generar un conjunto heterogéneo de productos irreversibles

denominados productos de glicosilación avanzada (Davis et al., 2001). Las reacciones de Maillard son importantes, ya que contribuyen al aroma y al sabor asociado a muchos alimentos procesados. Estas reacciones resultan importantes asimismo porque pueden afectar a la alergenicidad de proteínas alimentarias. Las modificaciones inducidas en esta reacción pueden hacer que ciertos alergenos formen agregados de alto peso molecular, los cuales unen IgE de forma más efectiva y resultan más resistentes a la digestión gástrica que los alergenos sin modificar (Mills et al., 2009). En estudios realizados con alergenos de cacahuete se demostró que los extractos de cacahuete tostado unen IgE de individuos alérgicos a un nivel significativamente mayor que los extractos de cacahuete crudo. El efecto observado se atribuyó, al menos en parte, a la reacción de Maillard (Maleki et al., 2000b; Chung and Champagne, 2001; Chung et al., 2003). Estos estudios demostraron que los alergenos Ara h 1 y Ara h 2 en cacahuete tostado son menos solubles, menos digeribles y unen más IgE que en el cacahuete crudo.

Modificación genética

La biotecnología está siendo empleada de diferentes maneras para aumentar los efectos positivos de los alimentos y reducir o abolir los efectos negativos. Por ejemplo, la ingeniería genética se puede aplicar para reducir la alergenicidad de un alimento determinado mediante la reducción o la eliminación de un alergeno particular (Lehrer, 2004). Esta eliminación se podría conseguir interfiriendo en el flujo de información genética a nivel transcripcional o a nivel post-transcripcional. En este último caso se emplean estrategias como ARN antisentido, co-supresión o ARN interferente (Dodo et al., 2005). Los intentos iniciales se concentraron en la disminución de la expresión de proteínas alergénicas, tal y como se ha hecho con alergenos de arroz (Tada et al., 1996)

y de soja (Herman et al., 2003) utilizando experimentos de ARN antisentido. Con este abordaje se consiguió una reducción significativa de proteínas alergénicas. Los experimentos de ARN interferente para el silenciamiento post-transcripcional de genes han mostrado ser una estrategia eficiente para la inhibición de la expresión de proteínas en plantas. Esta tecnología se ha aplicado en la supresión de la expresión de los alergenos mayores de manzana y cacahuete, Mal d 1 (Gilissen et al., 2005) y Ara h 2 (Dodo et al., 2005) respectivamente. También se ha demostrado que dos isoformas del alergeno de tomate Lyc e 3 (LTP) pueden ser suprimidas en hojas, así como en el fruto. El silenciamiento de ambas isoformas por medio de la tecnología del ARN interferente mostró una supresión casi completa del ARN mensajero que codifica para Lyc e 3 y de la expresión de la proteína (Le et al., 2006). Estos resultados fueron confirmados mediante pruebas cutáneas (Lorenz et al., 2006).

Alergenos alimentarios modificados mediante procesos tecnológicos

Enzimáticos

Actualmente los preparados que pueden ser considerados como hipoalergénicos son las fórmulas preparadas mediante hidrólisis enzimática y las fórmulas elementales, que están constituidas por aminoácidos libres (Bahna, 2008). Ambas pueden derivar de diferentes fuentes como leche de vaca o soja y se clasifican por el grado de hidrólisis como producto “exhaustivamente” o “parcialmente” hidrolizado (Høst y Halken, 2004). Los hidrolizados de leche han sido comercializados con éxito durante más de 50 años. Presentan un potencial alergénico reducido y se suelen emplear junto con otros

ingredientes hipoalergénicos en el desarrollo de productos diseñados para niños alérgicos a leche de vaca (Clemente, 2000). En la obtención de fórmulas hipoalergénicas, se deben caracterizar las propiedades del producto mediante técnicas bioquímicas y la reducción de la alergenicidad se debe constatar mediante experimentos *in vitro* usando técnicas como pruebas inmunoelectroforéticas, ELISA, inhibición de CAP, etc (Høst y Halken, 2004). Dichos experimentos deberían determinar el perfil de los pesos moleculares de los péptidos tras la hidrólisis y el potencial alergénico de los mismos (American Academy of Pediatrics, 2000). Van Beresteijn et al (1994) determinaron que la masa molecular mínima de los péptidos derivados de suero de leche necesaria para producir una respuesta mediada por IgE era de 3 a 5 kDa. Van Hoeyveld et al (1998) establecieron esta masa molecular mínima entre 0,9 y 1,4 kDa. Por otro lado, es necesario realizar pruebas *in vivo*, tales como pruebas cutáneas y provocaciones orales. La recomendación actual es que las fórmulas hipoalergénicas de leche de vaca deberían ser toleradas por al menos un 90% de niños alérgicos a este alimento. Algunos productos: exhaustivamente hidrolizados y basados en aminoácidos cumplen dicho criterio (Høst y Halken, 2004).

La Academia Americana de Pediatría estableció que si la fórmula sustitutiva de leche que se está probando no deriva de proteínas de leche, dicha fórmula se debe evaluar en niños con alergia clínica al alimento concreto (American Academy of Pediatrics, 2000). Por ejemplo, los hidrolizados de soja tienen una larga historia como fórmulas alternativas para niños alérgicos y aunque no sea un preparado del todo hipoalergénico, se puede utilizar en niños con alergia mediada por IgE a leche, especialmente después de los 6 meses de edad (American Academy of Pediatrics, 1998). También, en los últimos años se ha desarrollado una fórmula basada en hidrolizado exhaustivo de proteínas de arroz (Risolac; Heinz, Plada, Italia) como una fuente

alternativa de proteínas para niños con alergia a leche de vaca. Esta fórmula fue probada en un estudio multicéntrico en 100 niños alérgicos y los experimentos *in vitro* mostraron que un 73% de los sueros empleados reconocieron proteínas del extracto de arroz sin tratar, mientras que solo un 6% reconocieron proteínas del hidrolizado. Las provocaciones orales con la fórmula no produjeron ninguna reacción (Fiochi et al., 2006).

Las proteínas de origen vegetal están encontrando aplicación comercial en alimentos como alternativa a las fuentes de proteínas animales. Muchos estudios han mostrado el interés de los hidrolizados de origen vegetal como alimentos funcionales y suplementos del sabor. El efecto que la hidrólisis enzimática ejerce sobre la alergenicidad de soja ha sido ampliamente estudiado (American Academy of Pediatrics, 1998) y más recientemente ha sido evaluado en garbanzo y lupino (Clemente et al., 1999a; Sormus de Castro Pinto et al., 2009).

Las primeras enzimas proteolíticas utilizadas en la industria alimentaria fueron proteasas de origen animal, si bien cada vez están adquiriendo mayor importancia las de origen bacteriano o fúngico (Fritsché, 2009). La enzima Alcalasa es una endoproteasa de *Bacillus licheniformis* y la Flavorzima es una exoproteasa de *Aspergillus oryzae* con una actividad endoproteasa casi residual. Ambas son enzimas aptas para su uso en alimentos (Novozyme, Bagsvared, Denmark). Alcalasa y Flavorzima son enzimas que producen hidrolizados proteicos con un grado de hidrólisis mayor que el conseguido con papaína, tripsina o α-quimotripsina en un estudio realizado en guisante (Humiski y Aluko, 2007). La Flavorzima es una enzima que puede utilizarse en la eliminación del amargor en hidrolizados parciales (Clemente et al., 1999c). En general, la hidrólisis enzimática se desarrolla bajo condiciones suaves de pH (6-8) y temperatura (40-60ºC), evitando así las condiciones extremas necesarias en otros tratamientos, como los

químicos. Clemente et al (1999a) emplearon Alcalasa y Flavorzima de manera individual y secuencial con extracto de garbanzo. Los resultados demostraron que la hidrólisis secuencial con Alcalasa y Flavorzima (endo-exoproteasa) era el método más efectivo de los analizados para la reducción del reconocimiento de alergenos de garbanzo.

En general, los hidrolizados deben someterse a modificaciones después del proceso de hidrólisis, como la ultrafiltración para eliminar los péptidos residuales de alto peso molecular o procedimientos para la corrección del sabor amargo que suele caracterizar a los hidrolizados proteicos debido al contenido de aminoácidos hidrofóbicos (Clemente, 2000).

Tratamientos térmicos y de presión

La alergia a arroz es importante en Asia donde se ha realizado un gran esfuerzo en el desarrollo de variedades hipoalergénicas. En un tipo de arroz presurizado se logró disminuir en un 95% el contenido de albúminas y globulinas mediante un tratamiento de presión. Este arroz tratado fue administrado a 7 niños con dermatitis atópica e IgE específica a arroz dando lugar a una mejora de las reacciones cutáneas después de la introducción del arroz hipoalergénico durante 4 semanas (Juji et al., 1999). Otras variedades de arroz hipoalergénico desarrolladas en Asia son AFT-R 1, sometido a hidrólisis alcalina, HRS-1, hidrolizado enzimáticamente mediante actinasa y un tipo de arroz transgénico en el que se ha silenciado la expresión de los alergenos de 14 a 16 kDa (Fiocchi et al., 2004).

Experimentos realizados en semilla de lupino han demostrado que los tratamientos como el calentamiento en microondas, la cocción o la extrusión no tienen ningún efecto en sus alergenos, sin embargo un tratamiento de presión y calor: el

tratamiento con autoclave a 2,56 atmósferas (138ºC) durante 30 minutos, produce una disminución del reconocimiento de proteínas alergénicas en el extracto proteico por parte de los anticuerpos IgE de sueros de pacientes sensibilizados a lupino (Álvarez-Álvarez et al., 2005). Otro tratamiento tecnológico, la denominada Despresurización Instantánea Controlada (DIC®) tiene efectos similares en semillas de lupino a los obtenidos con autoclave (Guillamón et al., 2008). La tecnología DIC® fue desarrollada por Allaf et al en 1989 en la Universidad de La Rochelle, Francia. Este procedimiento aplica una despresurización instantánea para modificar la textura del material e intensificar la funcionalidad de los alimentos. El tratamiento DIC® consiste en introducir el material a tratar con un volumen de agua prefijado en una cámara donde es sometido a presión (hasta 8 bares) y altas temperaturas (hasta 170ºC) durante un período de tiempo corto (segundos) (Figura 1). El tratamiento térmico finaliza con una caída brusca de presión, provocada por la apertura instantánea de una válvula ubicada entre la cámara y un tanque de gran volumen a presión reducida (50 mbar). La caída de presión provoca una vaporización del agua del producto y una modificación de la textura del mismo adquiriendo una textura porosa. Este tratamiento termo-mecánico también produce la descontaminación microbiana.

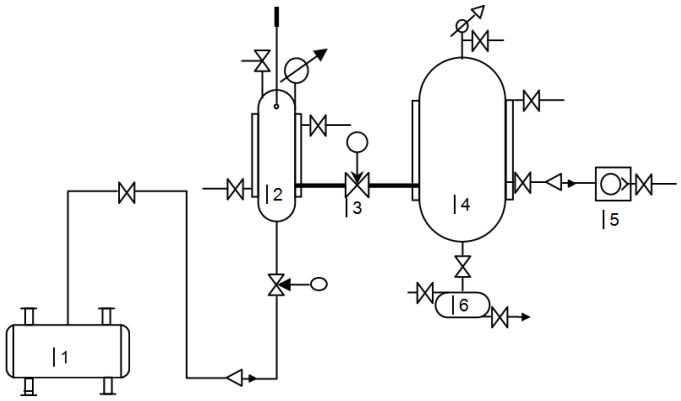

Figura 1. Representación del diagrama de trabajo de un reactor DIC® (1: caldera; 2: reactor DIC®; 3: válvula de descompresión; 4: tanque de vacío; 5: bomba de vacío; y 6: tanque de condensación).

La cocción, por su parte, es un tratamiento térmico ampliamente utilizado en la preparación de muchos alimentos entre ellos leguminosas como el garbanzo y la lenteja. En otras leguminosas, como el cacahuete, la forma de preparación culinaria de esta semilla en países como China se basa en la cocción (Beyer et al., 2001). Ésta puede tener diversos efectos en la alergenicidad de los alimentos; en un estudio con pescado se demostró una reducción parcial en la actividad alergénica del pescado hervido, principalmente a expensas de la termolabilidad de los alergenos de peso molecular superior a 40 kDa (Bernhisel-Broadbent et al., 1992). Mientras, otros estudios han constatado la elevada estabilidad de la actividad alérgica de alimentos como gamba hervida (Daul et al., 1988).

Tratamientos de pulsos eléctricos, luz ultravioleta e irradiación

Algunos de los tratamientos que se emplean en la eliminación de los microorganismos contaminantes de alimentos, como los pulsos eléctricos, pulsos de luz ultravioleta o irradiación gamma, han sido evaluados en relación con su efecto sobre la alergenicidad de alimentos. Chung et al (2008) y Yang et al (2011) estudiaron el efecto de los pulsos de luz ultravioleta en la alergenicidad de un extracto proteico de cacahuete. Ambos grupos encontraron una reducción de algunos de los alergenos de cacahuete después del tratamiento con luz ultravioleta, aunque se observó un aumento de agregados proteicos. Recientemente se ha comprobado que los tratamientos de pulsos eléctricos no afectan significativamente a la estructura secundaria de los alergenos Ara h 2 y Ara h 6 de cacahuete, ni a los alergenos Mal de 3 y Mal de 1 de manzana (Johnson et al., 2010). También se ha comprobado que el tratamiento de irradiación gamma solo o seguido de otros tratamientos con autoclave a 1 atm durante 15 y 30 minutos sobre semillas de

almendra, anacardo y nuez no tiene ningún efecto relevante sobre las proteínas de estos frutos secos (Su et al., 2004).

OBJETIVOS

El objetivo general del presente trabajo de investigación es analizar los efectos de los tratamientos enzimáticos, térmicos y de presión sobre la alergenicidad de alimentos, así como las modificaciones que algunos de estos tratamientos producen en la estructura secundaria y la solubilidad de las proteínas alimentarias. Además se aborda el estudio de la reactividad cruzada a nivel de anticuerpos IgE con relevancia clínica.

Los objetivos específicos son:

1. Estudiar los efectos que producen los tratamientos de hidrólisis enzimática con endo- y exopeptidasas en la alergenicidad de lenteja y cacahuete.
2. Evaluar los efectos de los tratamientos térmicos, autoclave y Despresurización Instantánea Controlada (DIC®) en el reconocimiento de proteínas de leguminosas por anticuerpos IgE.
3. Evaluar los cambios producidos en la alergenicidad, solubilidad y estructura secundaria de las proteínas de cacahuete debidos al procesamiento mediante presión.
4. Estudiar la posible implicación del procesamiento térmico en la alergenicidad de un producto dietético.
5. Evaluar la relevancia clínica de la reactividad cruzada entre miembros de la familia *Leguminosae* y entre alimentos implicados en el síndrome látex-frutas.

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Influence of Enzymatic Hydrolysis on the Allergenicity of Roasted Peanut Protein Extract

Beatriz Cabanillas[a] Mercedes M. Pedrosa[b] Julia Rodríguez[a] Mercedes Muzquiz[b]
Soheila J. Maleki[c] Carmen Cuadrado[b] Carmen Burbano[b] Jesús F. Crespo[a]

[a]Servicio de Alergia, Instituto de Investigación Hospital 12 de Octubre (i+12), and [b]Departamento de Tecnología de Alimentos, SGIT-INIA, Madrid, Spain; [c]US Department of Agriculture, Agriculture Research Service, Southern Regional Research Center, New Orleans, La., USA

Key Words
Enzymatic hydrolysis · Food allergy · Legumes · Roasted peanut

Abstract
Background: Peanut allergy is recognized as one of the most severe food allergies. Some studies have investigated the effects of enzymatic treatments on the in vitro immunological reactivity of members of the Leguminosae family, such as the soybean, chickpea and lentil. Nevertheless, there are only a few studies carried out with sera from patients with a well-documented allergy. ***Methods:*** Roasted peanut protein extract was hydrolyzed by the sequential and individual action of 2 food-grade enzymes, an endoprotease (Alcalase) and an exoprotease (Flavourzyme). Immunoreactivity to roasted peanut extract and hydrolyzed samples was evaluated by means of IgE immunoblot, ELISA and 2-dimensional electrophoresis using sera from 5 patients with a clinical allergy to peanuts and anti-Ara h 1, anti-Ara h 2 and anti-Ara h 3 immunoblots. ***Results:*** Immunoblot and ELISA assays showed an important decrease of IgE reactivity and Ara h 1, Ara h 2 and Ara h 3 levels in the first 30 min of hydrolyzation with Alcalase. In contrast, individual treatment with Flavourzyme caused an increase in IgE reactivity detected by ELISA at 30 min and led to a 65% inhibition of IgE reactivity at the end of the assay (300 min). Ara h 1 and the basic subunit of Ara h 3 were still recognized after treatment with Flavourzyme for 300 min. ***Conclusion:*** Hydrolysis with the endoprotease Alcalase decreases IgE reactivity in the soluble protein fraction of roasted peanut better than hydrolysis with the exoprotease Flavourzyme.

Introduction

Peanut allergy is recognized as one of the most severe food allergies due to its persistency and its often life-threatening nature [1]. The prevalence of peanut allergy appears to have increased in the western world during the last decades. An estimate of the prevalence of peanut allergy in children was 1.4% in 2008 compared with 0.8% in 2002 and 0.4% in 1997 in a self-reported population survey [2]. Currently, the only effective treatment for peanut allergy is avoidance of this nut in any form. However, total avoidance is difficult due to the widespread use of peanuts in the diet as an economical protein source.

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Correspondence to: Beatriz Cabanillas
Servicio de Alergia, Instituto de Investigación Hospital 12 de Octubre (i+12)
Avenida de Córdoba s/n
ES–28041 Madrid (Spain)
Tel. +34 91390 8660, E-Mail bcabanillas.hdoc@salud.madrid.org

Therefore, it is necessary to explore strategies designed to decrease the ability of peanuts to elicit dangerous allergic responses.

Several studies have evaluated the effects of enzymatic hydrolysis on the allergenicity and digestibility of food proteins [3–6]. Enzymatic hydrolysis is an efficient process for disrupting sequential and conformational epitopes [7]; therefore, protein hydrolysates could be an alternative to intact proteins in the development of special formulations for food-allergic patients [8]. However, depending on the type of enzymes used and the conditions of hydrolysis, peptides of different length may be obtained carrying more or less allergenicity [7, 9, 10]. Porcine trypsin/chymotrypsin is frequently used for producing hypoallergenic formulas, but proteases extracted from bacteria or of fungal origin are also increasingly used [7]. Two studies have analyzed the effects of the sequential action of an endoprotease from *Bacillus licheniformis* (Alcalase) and an exoprotease from *Aspergillus oryzae* (Flavourzyme) in chickpea [5] and lentil allergenicity [6]. These enzymes produce protein hydrolysates with a significantly higher degree of hydrolysis than papain, trypsin, and α-chymotrypsin [11]. In the chickpea and the lentil the sequential hydrolyzation with Alcalase and Flavourzyme produces a significant decrease in IgE recognition as shown with in vitro assays. Although further studies are needed to characterize the clinical relevance of these findings, this enzymatic procedure could be a suitable method to obtain less allergenic protein hydrolysates [5, 6].

The objective of this study was to investigate the effect of the individual and sequential action of Alcalase and Flavourzyme in the IgE-binding properties of the soluble protein fraction of roasted peanut (RP), using sera from patients with a clinical allergy to peanuts.

Material and Methods

Patients and Sera

Sera from 5 patients with a peanut allergy, confirmed on the basis of a positive double-blind placebo-controlled food challenge with peanut, were used in this study (table 1). An informed consent, approved by the Ethics Committee of our institution, was signed by the patients to carry out the study (Permission No. 0312150129). Subjects had a specific serum IgE level to peanuts ranging from 0.9 to 7.4 kU/l (median = 3.5 kU/l) as quantified by the fluorescent enzyme immunoassay (CAP-FEIA system, Phadia, Uppsala, Sweden).

A serum pool made with sera from the 5 peanut-allergic patients, and individual sera from patients 1, 2 and 4 were used in IgE immunodetection and ELISA assays.

Table 1. Clinical and immunological findings of 5 peanut-allergic patients

Patient No.	SPT, mm	CAP-FEIA, kU/l	Symptoms
1	4.5	3.5	A; DS; P; R; U
2	7	3.5	P
3	0	0.9	AE; DS; E; P; U
4	15.5	6.7	OAS
5	10	7.4	A; OAS; P; U

SPT = Skin prick testing; A = asthma; AE = angioedema; DS = difficulty swallowing; E = erythema; OAS = oral allergy syndrome; P = pruritus; R = rhinitis; U = urticaria.

Plant Material and Enzymatic Treatments

RPs (*Arachis hypogaea*, Virginia variety) obtained from Aperitivos Medina SL (Spain) were used in the study. Cleaned and free from foreign materials, they were milled to pass through a 1-mm sieve (Tecator, Cyclotec 1093, Sweden) and the resulting meal was defatted with n-hexane (34 ml/g of flour) for 4 h, shaken and air-dried after filtration.

Peanut flour was extracted according to the method reported by Cuadrado et al. [12] but using 0.05 M Tris-HCl (pH 8.0) plus 0.5 N NaCl as an extraction buffer. Flour was extracted twice at a 1:10 w/v ratio for 1 h at 4°C by stirring. The extract was centrifuged at 27,000 *g* for 20 min at 4°C, and the combined supernatants were dialyzed against distilled water for 48 h at 4°C using a dialysis membrane (Spectra/Por, Serva, Heidelberg, Germany) with a cutoff of 3.5 kDa, and then freeze-dried until use. The soluble protein content of the extract was determined by the Bradford dye-binding assay (Bio-Rad, Hercules, Calif., USA) using bovine serum albumin (Sigma, Mo., USA) as a standard.

Peanut protein extract was hydrolyzed according to Clemente et al. [5] with modifications. The food-grade enzymatic complexes used were Alcalase 2.4 L and Flavourzyme 1000 L (Novozyme A/S, Bagsvaerd, Denmark). Alcalase (2.4 L) is an endoprotease from *B. licheniformis*, with Subtilisin Carlsberg as the major enzymatic component, having a specific activity of 2.4 Anson units (AU) per gram. One AU is the amount of enzyme that digests hemoglobin under standard conditions at an initial rate that produces an amount of a trichloroacetic acid-soluble product which produces the same color with the Folin reagent as 1 mEq of tyrosine released per min. Flavourzyme (1000 L) is an exoprotease and endoprotease complex from *A. oryzae*, with an activity of 1.0 leucine aminopeptidase unit (LAPU) per gram. One LAPU is the amount of enzyme that hydrolyzes 1 mmol leucine-*p*-nitroanilide per min. According to the technical bulletin of Novozymes, Flavourzyme is mainly an exoprotease, the endoprotease activity being almost residual.

The hydrolysis was conducted in a 100-ml reaction vessel, equipped with a stirrer, a thermometer and a pH electrode. The protein extract was hydrolyzed batchwise with Alcalase and Flavourzyme by sequential and individual treatment. (1) Sequential treatment (480 min) was carried out with the initial hydrolysis (180 min) using Alcalase as endopeptidase and the second one

(300 min) using Flavourzyme as exopeptidase. Hydrolysis parameters for Alcalase were as follows: protein concentration (S) = 2%, enzyme to substrate ratio (E/S) = 0.4 AU/g of protein, temperature (T) = 50°C, pH 8.0. Flavourzyme hydrolysis parameters were: S = 2%, E/S = 100 LAPU/g of protein, T = 50°C, pH 7.0. (2) Individual treatment using Alcalase was developed during 150 min. Hydrolysis parameters for Alcalase were: S = 2%, E/S = 0.2 AU/g of protein, T = 50°C, pH 8.0. (3) Individual treatment using Flavourzyme was developed during 300 min. Flavourzyme hydrolysis parameters were: S = 2%, E/S = 100 LAPU/g of protein, T = 50°C, pH 7.0. Samples were withdrawn at certain time intervals and proteases in the aliquots were inactivated by heating at 80°C for 20 min.

The degree of hydrolysis (DH), defined as the percentage of peptide bonds cleaved, was measured by determination of the free amino groups by reaction with trinitrobenzene sulfonic acid (TNBS; Sigma) using leucine as the standard, according to the method of Adler-Nissen [13]. The total number of amino groups was determined by acid hydrolysis with 6 N HCl at 120°C for 24 h.

Immunodetection Assays

Protein Electrophoresis and IgE Immunoblot Experiments

SDS-PAGE was performed according to Laemmli [14]. Samples (20 μg protein per lane) were mixed with Laemmli sample buffer (Bio-Rad) and 2-mercaptoethanol (Bio-Rad) heated at 90°C for 10 min and electrophoresed in 4–20% Tris-HCl linear gradient precast gel (Bio-Rad). Proteins were visualized with Coomassie brilliant blue R-250 staining. Western blot was performed by electrophoretic transfer to nitrocellulose membranes (Whatman, Dassel, Germany). After blocking with 3% w/v nonfat milk, 0.1% v/v Tween 20 in PBS (pH 7.4, blocking buffer), the membranes were incubated overnight with the serum pool (1:40 dilution) or individual sera (1:10 dilution), washed, and then treated with mouse anti-human IgE mAb HE-2 ascitic fluid (1:5,000 dilution for 1 h) [15]. After washing, a goat anti-mouse IgG peroxidase-conjugated antibody (1:2,500 dilution for 1 h; Pierce, Ill., USA) was added. Detection of IgE-binding components was achieved by means of enhanced chemiluminescence using an ECL substrate according to the manufacturer's instructions (Amersham, UK). Membranes incubated directly with mouse anti-human IgE and goat anti-mouse IgG were used as negative controls.

Two-Dimensional Analysis

Two-dimensional analysis was carried out in the Proteomic Facility of the Instituto de Investigación Hospital 12 de Octubre (i+12). Lyophilized peanut extracts were dissolved in an appropriate volume of lysis buffer with 7 M urea, 2 M thiourea, and 2% CHAPS until completely resuspended. Samples were cleaned and desalted with the 2-D Clean-Up Kit (Amersham). Proteins were dissolved for at least 5 min in loading buffer containing 7 M urea, 2 M thiourea, 2% w/v CHAPS, 2% v/v IPG buffer, pH 3–11 NL, and 65 mM DTT. Immobiline DryStrip gel, pH 3–11 NL, 7 cm, was rehydrated in DeStreak Rehydration Solution (Amersham) and 2% IPG Buffer, pH 3–11. Seventy micrograms of protein were applied by cup loading and focused using the IPGphor 3 electrophoresis unit (GE Healthcare) at 20°C to reach 14 kV-h, with a maximum voltage of 1,200 V. For the second dimension, the IPG strips were reduced for 15 min in SDS equilibration buffer (6 M urea, 50 mM Tris-HCl, pH 8.8, 20% glycerol, 10% SDS, 1% w/v DTT, and a trace of bromophenol blue), and proteins were alkylated in the same buffer containing 2.5% w/v iodoacetamide instead of DTT for 15 min. Subsequent SDS-PAGE separation was performed using a mini PROTEAN II cell (Bio-Rad, Munich, Germany). Two-dimensional gels were Coomassie blue stained following the manufacturer's instructions (Brilliant Blue G-Colloidal Concentrate, Electrophoresis Reagent, B2025-1EA, Sigma-Aldrich). Proteomic MALDI-TOF/TOF analysis of Coomassie blue-stained spots of interest was carried out in the UCM-PCM Proteomic Facility, a member of the ProteoRed network. Spots were washed, reduced, alkylated and digested as previously described [16]. Mass spectrometry analyses were performed in a MALDI-TOF spectrometer 4800 Proteomics Analyzer (PerSeptives Biosystems, Framingham, Mass., USA) [16]. Proteins for which peptide mass fingerprints provided an ambiguous identification were subjected to MS/MS sequencing analyses [16]. For protein identification, the nonredundant National Center for Biotechnology Information database was searched using MASCOT 2.1 (www.matrixscience.com) through the Global Protein Server v3.6 from Applied Biosystems. Identifications were accepted as positive when at least 5 matching peptides and at least 20% of the peptide coverage of theoretical sequences matched, setting the probability score at $p < 0.05$.

ELISA

Specific IgE binding to RP extract and hydrolyzed samples was assessed by means of indirect ELISA with the serum pool from the 5 patients with a clinical allergy to peanuts. Polystyrene microtiter plates (Costar 3590, Corning, N.Y., USA) were coated with 100 μl/well of extract (30 μg/ml in 0.01 M PBS) and incubated at 4°C overnight. Wells were washed with PBS containing 0.5% Tween 20 (v/v) and blocked with PBS containing 3% nonfat milk (w/v) and 0.1% Tween 20 (200 μl/well). Plates were incubated overnight with the serum pool (100 μl/well, 1:10 dilution) and binding of IgE was detected by incubation for 1 h with mouse anti-human IgE mAb HE-2 ascitic fluid (100 μl/well, 1:5,000 dilution) [15] followed by goat anti-mouse IgG peroxidase-conjugated (100 μl/well, 1:2,500 dilution for 1 h, Pierce). Finally, the peroxidase reaction was developed with 50 μl of peroxidase substrate buffer (Dako, Glostrup, Denmark). After 30 min, the reaction was stopped with 50 μl of 4 N H_2SO_4, and the optical density (OD) was measured at 492 nm. The following negative controls were included: (1) wells were coated with nonfat milk (instead of peanut samples) and incubated with the serum pool from the 5 patients following the process described above and (2) wells were coated with peanut samples and incubated directly with mouse anti-human IgE and goat anti-mouse IgG.

All tests were performed in triplicate. The formula, mean (OD) + 3 × SD, was calculated for each negative control and the highest value was considered as a cutoff point for positivity.

The percentage of the decrease in IgE reactivity was calculated with the formula: $(1 - A_H/A_N) \times 100$, where A_H is the absorbance value obtained by hydrolyzed samples and A_N is the absorbance value of the protein extract sample.

Anti-Ara h 1, Anti-Ara h 2 and Anti-Ara h 3 Immunoblots

RP before and after sequential and individual treatment with Alcalase and Flavourzyme was assessed for Ara h 1, Ara h 2 and Ara h 3 profiles. Samples (20 μg protein per lane) underwent elec-

trophoresis and were transferred. Membranes were preblocked for 1 h at room temperature (RT) in 5% w/v nonfat milk, 0.05% v/v Tween 20 in PBS. Chicken anti-Ara h 1 (1:10,000), chicken anti-Ara h 2 (1:8,000) and chicken anti-Ara h 3 (1:5,000) (custom-synthesized by Sigma Immunosys, The Woodlands, Tex., USA) were diluted in 5% w/v nonfat milk, 0.05% v/v Tween 20 in PBS and incubated with the membrane for 1 h at RT. The HRP-labeled antichicken IgY (1:100,000; Sigma Immunosys) was diluted in 2% w/v nonfat milk, 0.05% v/v Tween 20 in PBS and incubation time was 30 min at RT. Detection was achieved as described above.

Results

Protein Extract Hydrolysis

Peanut protein hydrolysates were obtained by: (1) sequential treatment with the endoprotease Alcalase (E/S = 0.4 AU/g) and the exoprotease Flavourzyme (E/S = 100 LAPU/g), (2) individual treatment with Alcalase (E/S = 0.2 AU/g) and (3) individual treatment with Flavourzyme (E/S = 100 LAPU/g). Figure 1 shows the hydrolysis curve of peanut protein extract over the indicated times. The x-axis shows the time of reaction and the y-axis shows the DH. Protein hydrolysis took place rapidly in the first 30 min; further, progression was slower. The combination of both enzymes yielded a DH of 69% at the end of the process (480 min). Individual treatment with Alcalase (0.2 AU/g) reached 17% DH after 150 min and individual treatment with Flavourzyme reached 29% DH after 300 min.

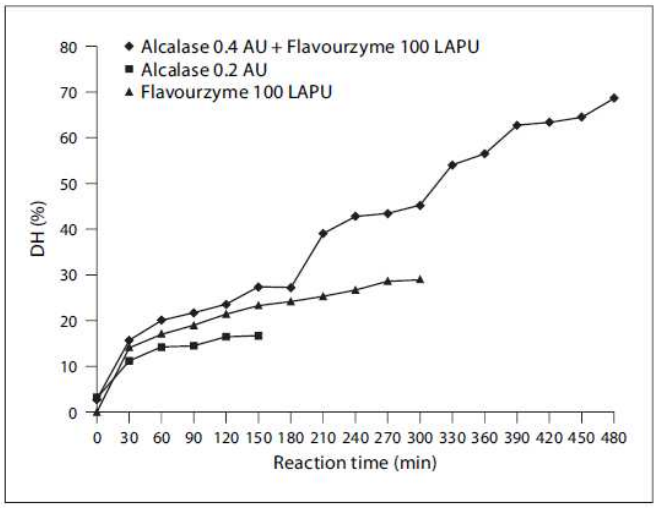


Fig. 1. Hydrolysis curves of peanut protein obtained by sequential and individual treatments with Alcalase and Flavourzyme. DH is defined as the percentage of peptide bonds cleaved.

Electrophoretic Characterization of Protein Hydrolysates

Figure 2a shows the SDS-PAGE protein patterns of RP before and after sequential treatment with Alcalase and Flavourzyme (fig. 2a1), and individual treatment with Alcalase (fig. 2a2) and Flavourzyme (fig. 2a3). Multiple bands can be seen in the RP sample with molecular weights between 10 and 63 kDa. Less stained bands but an increase of low molecular weight smears were observed after treatment with Alcalase 0.4 AU/g (fig. 2a1) and 0.2 AU/g (fig. 2a2) for 15 s. Hydrolysates with DH >16 and 11% (Alcalase 0.4 AU/g and 0.2 AU/g >30 min) showed a decrease of high-molecular-weight proteins. RP after individual treatment with Flavourzyme (fig. 2a3) showed fewer effects over the stained bands.

All samples were analyzed with IgE immunoblot using a serum pool from the 5 patients with a clinical allergy to peanuts. Figure 2b shows the IgE-binding protein patterns of RP before and after sequential treatment with Alcalase and Flavourzyme (fig. 2b1), and individual treatment with Alcalase (fig. 2b2) and Flavourzyme (fig. 2b3). RP showed a pattern of IgE-binding proteins in the range of 12–16 to 55 kDa. The overall IgE immunoreactivity was reduced after sequential endo- and exoprotease hydrolysis (480 min) and individual hydrolysis with Alcalase 0.2 AU/g (150 min). However, RP after treatment with Alcalase 0.2 AU/g (fig. 2b2) showed more IgE-binding proteins after hydrolysis during 15 s than Alcalase 0.4 AU/g (fig. 2b1). Individual treatment with Flavourzyme caused less reduction in IgE-binding proteins (fig. 2b3). A band of 22 kDa was still strongly recognized after treatment with Flavourzyme for 300 min. No bands were found in the negative controls (data not shown).

IgE reactivity to RP and the selected times of sequential and individual hydrolysis of RP with Alcalase and Flavourzyme were screened using 3 individual sera (patients 1, 2 and 4) (fig. 3). None of the sera recognized any RP proteins after sequential endo- and exoprotease hydrolysis (480 min). However, after individual treatment with Flavourzyme, the 3 sera recognized multiple bands. Proteins of 22 and 10 kDa were recognized by the 3 sera after hydrolysis during 300 min, and sera from patients 1 and 4 detected a 63- and a 30-kDa protein at this time of hydrolysis.

Two-Dimensional Analysis

In order to study the proteins still recognized by the sera after 5 h of hydrolysis with Flavourzyme, a 2-dimen-

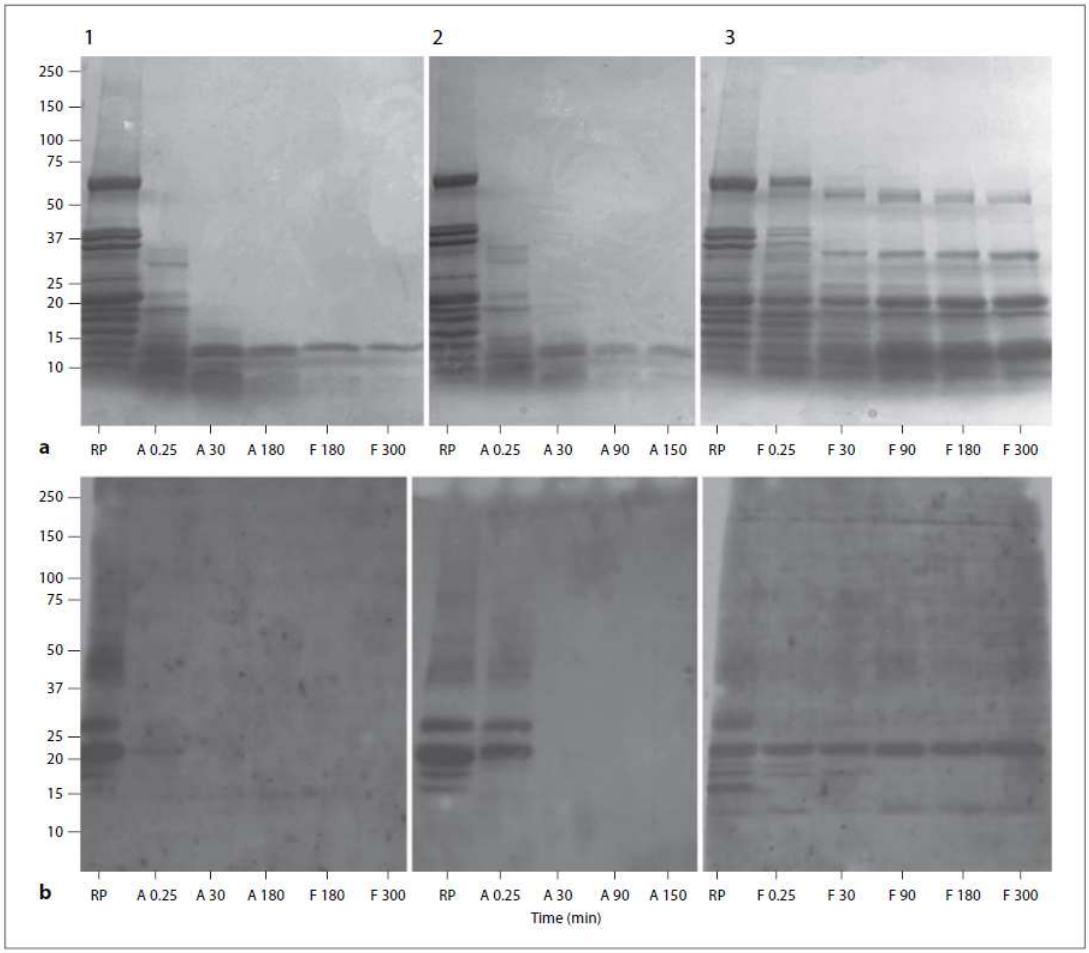


Fig. 2. SDS-PAGE (**a**) and Western blotting (**b**) of RP before and after sequential treatment with Alcalase 0.4 AU/g and Flavourzyme 100 LAPU/g (lane 1), individual treatment with Alcalase 0.2 AU/g (lane 2) and individual treatment with Flavourzyme 100 LAPU/g (lane 3), at the times indicated at the bottom of each figure. Western blotting was carried out with a serum pool from 5 patients with a clinical allergy to peanuts. A = Alcalase; F = Flavourzyme.

sional analysis of RP and RP after individual treatment with Flavourzyme during 300 min was carried out. Figure 4 shows the 2-dimensional protein staining of both samples and the Western blot of RP after individual treatment with Flavourzyme during 300 min, using the serum pool from the 5 peanut-allergic patients. Two-dimensional protein staining of RP showed several protein spots, whereas RP after individual treatment with Flavourzyme during 300 min showed spots of approximately 22 kDa and an isoelectric point (pI) ranging from 6 to 9.5 and spots of 15 kDa and pI between 3 and 9. Three spots of 22 kDa and pI ranging from 5 to 7 reacted with IgE antibodies. The spots were tryptic-digested in order to carry out the analysis by MALDI-TOF. The study of MALDI-TOF/MS using Mascot enabled us to match the 3 spots with the basic subunit of Ara h 3 (data not shown).

ELISA

In order to assess the IgE reactivity of RP hydrolysates, an ELISA was carried out using the serum pool from the 5 patients with a clinical allergy to peanuts. The positive cutoff point was 0.085 OD units, which was the highest value after applying the formula: mean (OD) + 3 × SD for each negative control. The ELISA results are summa-

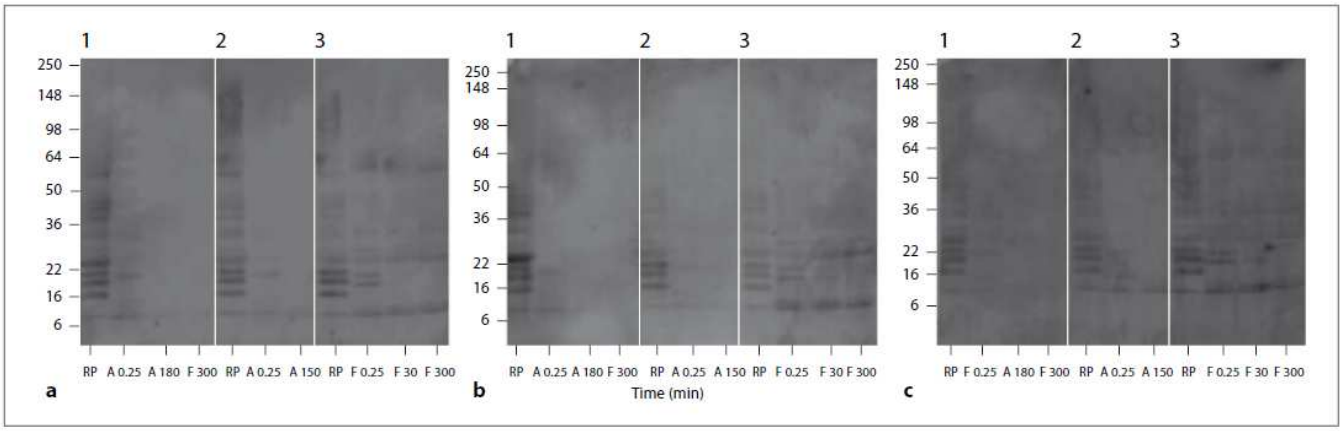


Fig. 3. Western blotting of RP before and after sequential treatment with Alcalase 0.4 AU/g and Flavourzyme 100 LAPU/g (lane 1), individual treatment with Alcalase 0.2 AU/g (lane 2) and individual treatment with Flavourzyme 100 LAPU/g (lane 3), at the times indicated at the bottom of each figure. Membranes were incubated with individual sera from 3 patients with a clinical allergy to peanuts: patient 1 (**a**), patient 2 (**b**) and patient 4 (**c**). A = Alcalase; F = Flavourzyme.

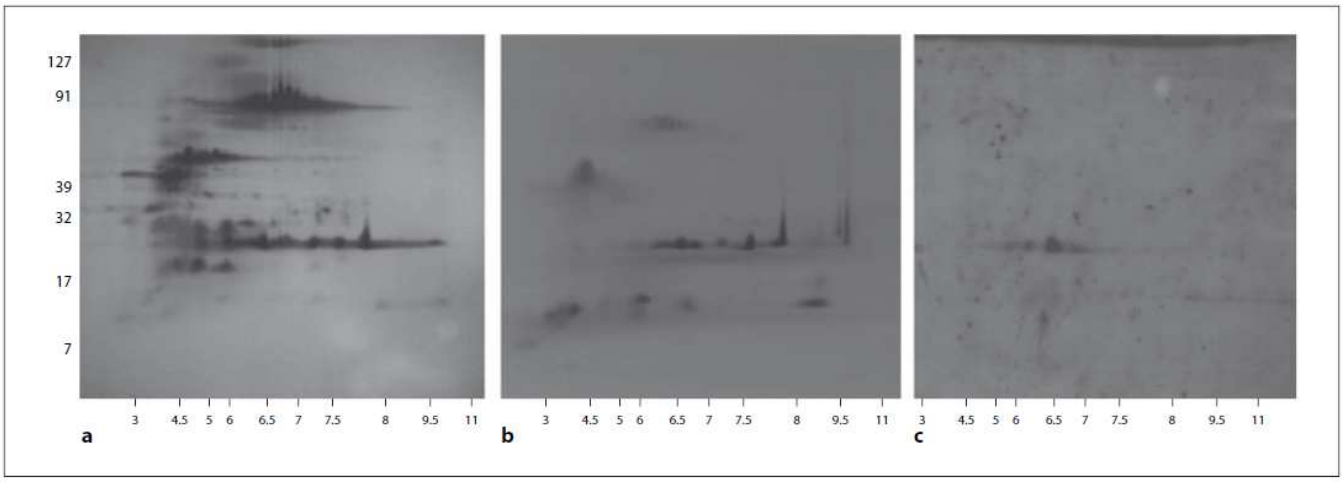
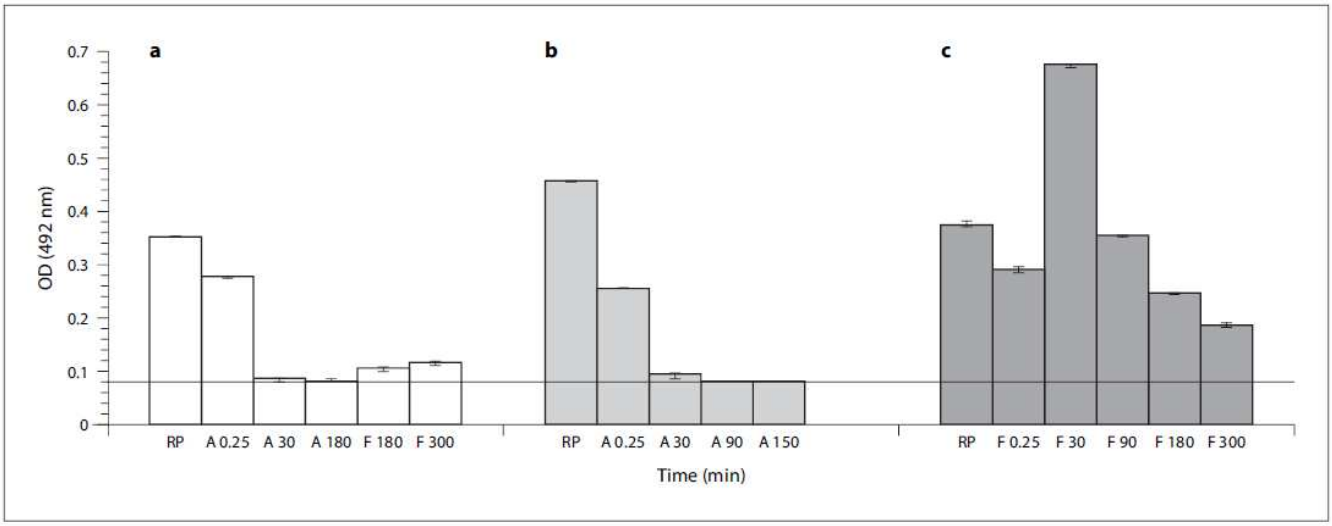


Fig. 4. Two-dimensional protein staining of RP (**a**), RP after individual treatment with Flavourzyme during 300 min (**b**), and Western blot of RP after individual treatment with Flavourzyme during 300 min (**c**), using a serum pool from 5 peanut-allergic patients.

rized in figure 5. Percentages of reduction in IgE reactivity are shown in the table annexed to figure 5. Hydrolysis with Alcalase/Flavourzyme sequentially and with Alcalase individually caused a higher loss of IgE reactivity in RP than hydrolysis with Flavourzyme individually. Alcalase was very effective in reducing the IgE reactivity of RP proteins since the enzyme at 0.4 and 0.2 AU/g led to a 100 and 98% reduction in IgE reactivity, respectively, in the first 30 min (DH 16 and 11%, respectively); the addition of Flavourzyme in the sequential assay caused a slight increase in IgE reactivity above the positive cutoff point. Individual treatment with Flavourzyme during 30 min caused an increase in IgE reactivity. Nevertheless, Flavourzyme led to a 65% reduction in IgE reactivity at the end of the assay (300 min).



Alcalase (0.4 AU) and Flavourzyme (100 LAPU)		
Time, min	reduction, %	DH, %
A 0.25	29	3
A 30	100	16
A 180	100	27
F 180	92	56
F 300	88	69

Alcalase (0.2 AU)		
Time, min	reduction, %	DH, %
A 0.25	54	3
A 30	98	11
A 90	100	14
F 150	100	17

Flavourzyme (100 LAPU)		
Time, min	reduction, %	DH, %
F 0.25	29	0
F 30	0	14
F 90	7	19
F 180	44	24
F 300	64	29

Fig. 5. Specific IgE to RP before and after sequential treatment with Alcalase 0.4 AU/g and Flavourzyme 100 LAPU/g (**a**), individual treatment with Alcalase 0.2 AU/g (**b**), and individual treatment with Flavourzyme 100 LAPU/g (**c**), at the times indicated at the bottom of each figure, using a serum pool from 5 peanut-allergic patients. The horizontal line indicates the cutoff point for positivity (0.085). Percentages of reduction in IgE reactivity and DHs are also shown. A = Alcalase; F = Flavourzyme.

Anti-Ara h 1, Anti-Ara h 2 and Anti-Ara h 3 Immunoblots

Specific anti-Ara h 1, anti-Ara h 2 and anti-Ara h 3 antibodies were used to identify Ara h 1, Ara h 2 and Ara h 3 molecules in RP before and after sequential and individual treatment with Alcalase and Flavourzyme. In figure 6, the Ara h 1 (63 kDa), Ara h 2 doublet bands (19 and 21 kDa), Ara h 3 (40 kDa) acidic subunit and Ara h 3 (23 kDa) basic subunit are indicated with arrows. There was a marked decrease in recognition of Ara h 1 (fig. 6a), Ara h 2 (fig. 6b) and Ara h 3 (fig. 6c) in RP after sequential endo- and exoprotease hydrolysis and individual hydrolysis with Alcalase. Individual treatment with Flavourzyme caused a decrease of Ara h 2 and the acidic subunit of Ara h 3 levels in the first 30 min of hydrolysis; after this time, both allergens were undetected. Ara h 1 levels decreased with increased hydrolysis time with Flavourzyme; however, it was still detected after 300 min of hydrolysis. The basic subunit of Ara h 3 was not affected by Flavourzyme hydrolysis.

Discussion

In this study an endoprotease (Alcalase) and an exoprotease (Flavourzyme) were used, both individually and sequentially to evaluate their effects in the IgE antibody reactivity to RP protein extract. RP was selected in this study because it has been recognized that it is more allergenic than raw peanut [17–22]. RP extract binds IgE from patients with peanut allergy at approximately 90-fold higher levels than that of raw peanuts, and the protein

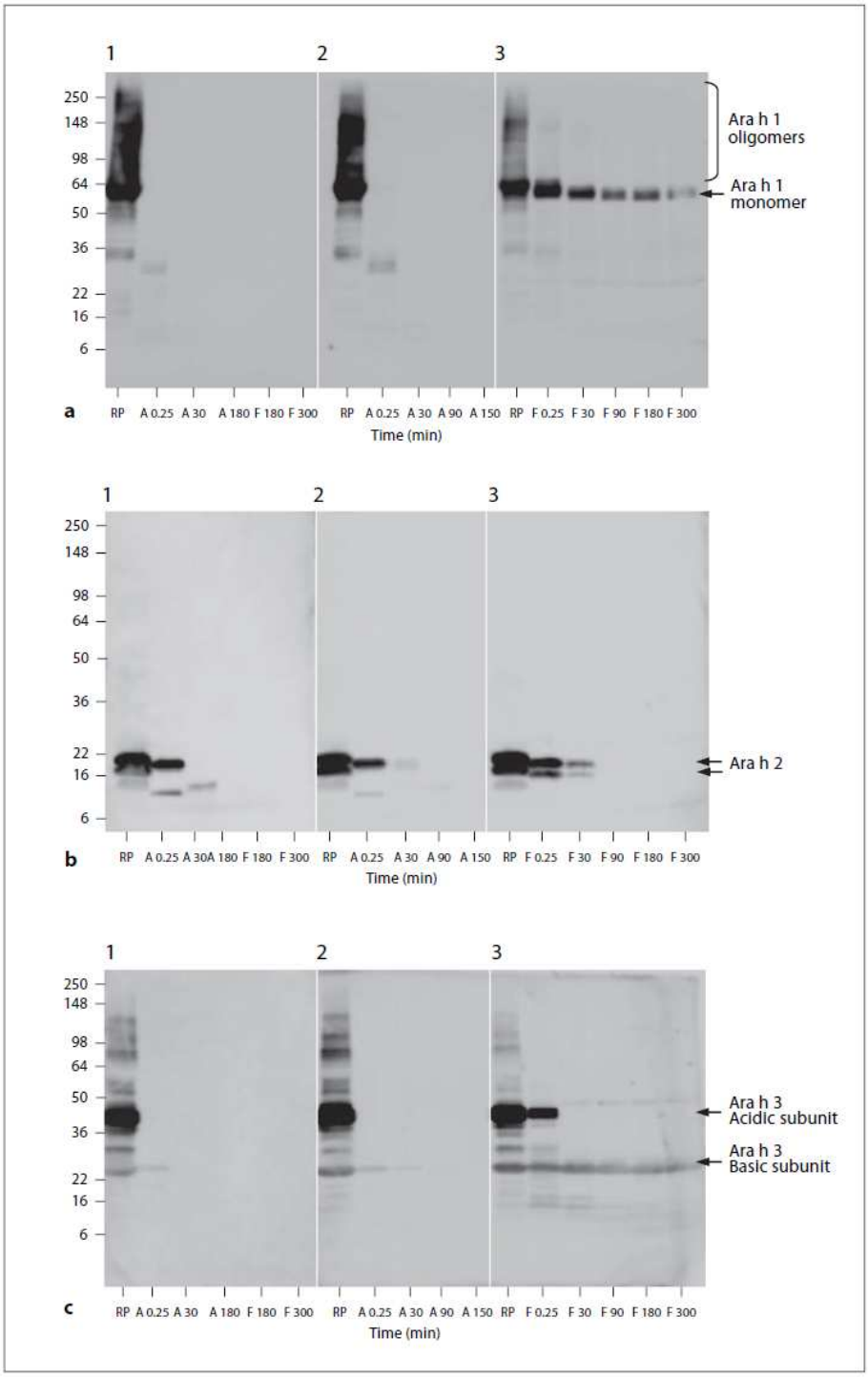


Fig. 6. Western blot analysis with anti-Ara h 1 (**a**), anti-Ara h 2 (**b**) and anti-Ara h 3 (**c**) antibodies. The Ara h 1, Ara h 2 and Ara h 3 profiles were assessed in RP before and after sequential treatment with Alcalase 0.4 AU/g and Flavourzyme 100 LAPU/g (lane 1), individual treatment with Alcalase 0.2 AU/g (lane 2), and individual treatment with Flavourzyme 100 LAPU/g (lane 3), at the times indicated at the bottom of each figure. A = Alcalase; F = Flavourzyme.

modifications induced by the Maillard reaction contribute to the observed effect [17, 20, 21].

Our results showed that individual treatment with Flavourzyme at 300 min reached a higher DH (29%) than individual treatment with Alcalase 0.2 AU/g (17%) at 150 min and similar to Alcalase 0.4 AU/g (27%) at 180 min; however, SDS-PAGE protein patterns were very different in both cases. In contrast to Flavourzyme hydrolysates, Alcalase hydrolysates showed no detectable stained proteins in SDS-PAGE. Similar results have been previously

reported for chickpeas [5]. Individual hydrolysis of chickpeas with Flavourzyme or Alcalase reached a similar DH (27%); however, Alcalase hydrolysates did not show visible electrophoretic bands in contrast to those obtained with Flavourzyme. An important decrease in stained proteins in SDS-PAGE during hydrolysis with Alcalase has been also reported for lentils [6].

Immunoblot and ELISA assays carried out with the serum pool from the 5 patients with a clinical allergy to peanuts showed a decrease of IgE reactivity in the first minute of hydrolyzation with Alcalase 0.4 AU/g (sequential treatment) and at 30 min with Alcalase 0.2 AU/g (individual treatment). After 30 min of hydrolysis, no bands were detected in immunoblotting and a 100% reduction in IgE reactivity was observed in ELISA. These results were confirmed by Western blot with individual sera. None of the sera recognized any RP proteins after sequential endo- and exoprotease hydrolysis. In contrast, individual treatment with Flavourzyme caused an increase in IgE reactivity detected by ELISA at 30 min. However, Flavourzyme led to a 65% decrease in IgE reactivity at the end of the assay (300 min). Clemente et al. [5] found that partially hydrolyzed chickpea proteins produced by individual treatment with Flavourzyme increased the IgE reactivity compared to the protein isolate. It has been hypothesized that new antigenic determinants could be found after the exposure of this legume to the exoprotease Flavourzyme, resulting in an increase of allergenicity. The most effective reduction of chickpea protein antigenicity was obtained by sequential treatment with Alcalase and Flavourzyme. In a study on lentils, sequential hydrolysis with Alcalase and Flavourzyme produced a decrease in IgE recognition when evaluated by in vitro assays using sera from patients with a clinical allergy to lentils [6].

Three spots of 22 kDa and pI ranging from 5 to 7 were still recognized after individual treatment with Flavourzyme for 300 min in Western blot using the serum pool. The spots reacting with IgE were identified as the basic subunits of Ara h 3 (11S globulin) by MALDI-TOF analysis. Although the basic subunit of Ara h 3 is considered a minor allergen, Restani et al. [23] suggested that it may have been a major allergen in a group of children allergic to peanuts in Italy. This finding was not in agreement with previous studies [24] indicating that allergenic epitopes occurred only in the acidic Ara h 3 subunit. However, other studies [25–27] have shown that Ara h 3 basic subunits may be important allergenic peptides. The resistance of the 11S globulin basic subunit to enzymatic hydrolysis has been reported previously in soybeans [28].

Three of the individual sera tested in Western blot recognized the basic subunit of Ara h 3 and also a 10-kDa band after individual treatment with Flavourzyme for 300 min. Sera from patients 1 and 4 detected a 63-kDa (Ara h 1) and a 30-kDa protein at this time point. These results highlight the importance of testing individual sera, since individual allergen recognition of specific sera might be diluted in a pool [6].

Specific anti-Ara h 1-, anti-Ara h 2- and anti-Ara h 3-binding experiments revealed that sequential endo- and exoprotease hydrolysis and individual hydrolysis with Alcalase decreased Ara h 1, Ara h 2 and Ara h 3 levels from the first minutes of hydrolysis. However, Ara h 1 and the basic subunit of Ara h 3 were not affected by Flavourzyme hydrolysis, confirming the results obtained in Western blot with pooled and individual sera.

Our results show that hydrolysis with Alcalase produces a decrease in IgE recognition in RP protein extract due to a decrease in the main peanut allergens. These enzymatically treated protein hydrolysates could constitute an alternative to intact proteins in the development of different products. Peanut protein isolates are used where bland and highly concentrated forms of peanut protein are desired, e.g. in bread and bakery goods [29]. Fortification of cereals with legumes, such as peanuts, has resulted in improving the nutritional quality of human dietary proteins [30]. Further studies are needed to evaluate the effects of these enzymes in the insoluble fraction of RP. Roasting can cause large changes in the biochemical characteristics of proteins due to the Maillard reaction. Proteins could form oligomers, become denatured, degraded, aggregated, crosslinked, fragmented and reassembled and these changes most often cause a reduction in solubility [31, 32].

In conclusion, although in vivo and ex vivo experiments will be necessary to evaluate the allergenicity of hydrolyzed peanut protein extract, the results in the present study show that hydrolysis with the endoprotease Alcalase decreases IgE reactivity in the soluble protein fraction of RP better than hydrolysis with the exoprotease Flavourzyme.

Acknowledgments

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RESEARCH ARTICLE

Effects of enzymatic hydrolysis on lentil allergenicity

Beatriz Cabanillas[1], Mercedes M. Pedrosa[2], Julia Rodríguez[1], Ángela González[1], Mercedes Muzquiz[2], Carmen Cuadrado[1], Jesús F. Crespo[1] and Carmen Burbano[2]

[1] Servicio de Alergia, Hospital Universitario 12 de Octubre, Madrid, Spain
[2] Departamento de Tecnología de Alimentos, SGIT-INIA, Madrid, Spain

Enzymatic hydrolysis and further processing are commonly used to produce hypoallergenic dietary products derived from different protein sources, such as cow's milk. Lentils and chickpeas seem to be an important cause of IgE-mediated hypersensitivity in the Mediterranean area and India. Some studies have investigated the effects of enzymatic treatments on the *in vitro* immunological reactivity of members of the *Leguminosae* family, such as soybean, chickpea, lentil, and lupine. Nevertheless, there are only a few studies carried out to evaluate the effect on IgE reactivity of these food-hydrolysis products with sera from patients with well-documented allergy to these foods. In this study, lentil protein extract was hydrolyzed by sequential action of an endoprotease (Alcalase) and an exoprotease (Flavourzyme). Immunoreactivity to raw and hydrolyzed lentil extract was evaluated by means of IgE immunoblotting and ELISA using sera from five patients with clinical allergy to lentil. The results indicated that sequential hydrolysis of lentil results in an important proteolytic destruction of IgE-binding epitopes shown by *in vitro* experiments. However, some allergenic proteins were still detected by sera from four out of five patients in the last step of sequential hydrolyzation.

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1 Introduction

In recent years, legume seed proteins are gaining importance as beneficial food ingredients and their consumption is recommended by health organizations and dieticians [1] Among plant proteins, soybean has been the most widely used source, but other legumes are also important [2]. Lentil consumption is increasing, mainly in developing countries as a vehicle for mineral biofortification [3].

Legumes are among the most common allergenic foods causing allergic reactions in children in the Mediterranean area [4–6]. A previous study showed that lentils ranked fourth, after hen eggs, fish, and cow's milk, as a cause of food allergy in children from Spain [7]. Two lentil allergens have been described so far. A major lentil allergen Len c 1 has been isolated and identified as a 48 kDa vicilin; its processing fragments, corresponding to subunits 12–16 and 26 kDa are also relevant lentil IgE-binding proteins [8, 9]. Len c 2 has been isolated and identified as a 66 kDa seed-specific biotinylated protein [8].

A number of studies have evaluated the effects of enzymatic hydrolysis on the allergenicity and digestibility of food proteins. Cow's milk hydrolysate formulae have been developed to decrease or eliminate its allergenicity [10, 11]. Changes in allergenicity of enzymatically hydrolyzed legumes have been evaluated mainly in soybean [12, 13] and peanut [13]. Studies with other members of the *Leguminosae* family are scarce. Clemente *et al.* [14] evaluated the reduction of immunoreactivity to chickpea allergens during extensive hydrolysis generated by the sequential action of an endoprotease (Alcalase) and an exoprotease (Flavourzyme). A decrease in IgE-binding capacity by more than 90% was detected using sera from six patients who reported reactions after chickpea ingestion. Moreover, this sequential

Correspondence: Beatriz Cabanillas, Servicio de Alergia, Hospital Universitario 12 de Octubre, Avda. de Córdoba s/n, 28041 Madrid, Spain
E-mail: bcabanillas.hdoc@salud.madrid.org
Fax: +34-91-3908261

Abbreviations: **AU**, Anson units; **DH**, degree of hydrolysis; ***E/S***, enzyme substrate ratio; **LAPU**, leucine aminopeptidase unit

hydrolysate is considered an interesting material as food additive due to its high protein quality [15]. Sormus de Castro Pinto *et al.* [16] investigated the effects of pepsin and trypsin on the *in vitro* reactivity of 11S globulin of lupine, chickpea, and lentil using rabbit antiserum for these proteins. They obtained a large amount of short peptides and free amino acids after enzymatic treatment. The assessment of different enzyme activities to decrease allergenicity seems to be important; moreover, these studies should be carried out with sera from patients with documented clinical allergy to the source food [17].

Enzymatic protein hydrolysates constitute an alternative to intact proteins in the development of special formulations designed to provide nutritional support to specific population groups with different needs, such as infants, elderly, and food-allergic patients. In addition, protein hydrolysates show technological advantages. The combination of extensive enzymatic treatment coupled with post hydrolysis food processing procedures, such as heat treatment, ultrafiltration, *etc.*, is considered the most effective way to obtain protein products with an added high value for human nutrition and reduced allergenicity [18].

The objective of this study was to investigate the effect of sequential action of Alcalase (endoprotease) and Flavourzyme (exoprotease) in lentil allergenicity using sera from patients with well-documented lentil allergy. The potential utility of these lentil protein hydrolysates as hypoallergenic ingredients in food formulae is considered.

2 Materials and methods

2.1 Patients and sera

Sera from five patients with a convincing history of recent severe systemic anaphylaxis after isolated lentil ingestion were used in this study (Table 1). An informed consent, approved by the Ethic Committee of our Institution (Permission No. 0312150129) was signed by the patients to carry out the study. All subjects had a positive skin prick test response and a specific serum IgE level to lentil ranging from 1.24 to 30.7 kU/L (median=7.78 kU/L), as quantified by the CAP-fluorescent enzyme immunoassay System (Phadia, Uppsala, Sweden). A serum from a patient with a specific IgE to *Anisakis* spp. (9.09 kU/L), a specific IgE $<$ 0.35 kU/L to lentil, and a total serum IgE value of 53.4 kU/L was used as a negative control.

A serum pool made with sera from the five lentil-allergic patients and individual sera from all patients were used in the IgE immunodetection and ELISA assays.

Table 1. Clinical and immunologic findings of five patients with a convincing history of severe systemic anaphylaxis to lentil

Patient no.	Lentil		
	SPT (mm)	CAP-FEIA (kU/L)	Symptoms
1	13.00	1.24	A;G;H;P
2	8.50	20.20	A;AE;DS;G;GI;T;OAS;P;U
3	5.00	6.13	A;DY;P
4	16.50	30.70	A;O;OAS;P;R;U
5	12.00	7.78	A;AE;DS;G;GI;OAS;T

SPT, skin prick testing; A, asthma; AE, angioedema; DS, difficulty swallowing; DY, dysphonia; G, general malaise; GI, gastrointestinal symptoms; H, hypotension; O, ocular symptoms T, tongue swelling, OAS, oral allergy syndrome; P, pruritus; R, rhinitis; U, urticaria.

2.2 Plant material and enzymatic treatments

Lentil seeds (*Lens culinaris* cv Guareña) obtained from the *Servicio de Investigación y Tecnología Agraria* (Valladolid, Spain) were used for the study. Seeds, cleaned and free from foreign materials, were ground to pass through a 1-mm sieve (Cyclotec 1093,Tecator, Sweden), and the flour was defatted with *n*-hexane (34 mL/g of flour) for 4 h, shaken and air-dried after filtration.

The defatted lentil flour was extracted according to the method reported by Cuadrado *et al.* [19],] but using 0.1 M PBS (pH 7.4) buffer plus 0.15 M NaCl containing a 1% PVP (Calbiochem, Darmstadt, Germany) as extraction buffer. The flour was extracted twice at a 1:10 w/v ratio for 1 h at 4°C by stirring. The extract was centrifuged at 27 000 $\times$ *g* for 20 min at 4°C, and the combined supernatants were dialysed against distilled water for 48 h at 4°C and freeze-dried until use. The soluble protein content of the extract was determined by Bradford dye-binding assay (Bio-Rad, CA, USA), using bovine serum albumin (Sigma, MO, USA) as a standard.

The lentil protein extract was hydrolyzed according to the method reported by Clemente *et al.* [14] with modifications. The enzymatic complexes used were Alcalase 2.4 L and Flavourzyme 1000 L (Novozyme A/S, Bagsvaerd, Denmark). Alcalase (2.4 L) is an endopeptidase from *Bacillus licheniformis*, with Subtilisin Carlsberg as the major enzymatic component, having a specific activity of 2.4 Anson units (AU) *per* gram. One AU is the amount of enzyme that digests hemoglobin under standard conditions at an initial rate that produces an amount of trichloroacetic acid-soluble product which gives the same colour with the Folin reagent as one mequiv of tyrosine released *per* minute. Flavourzyme (1000 L) is an exopeptidase and endoprotease complex with an activity of 1.0 leucine aminopeptidase unit (LAPU) *per* gram. One LAPU is the amount of enzyme that hydrolyzes 1 mmol leucine-*p*-nitroanilide *per* minute.

The hydrolysis was conducted in a 100-mL reaction vessel, equipped with a stirrer, thermometer, and pH electrode. The protein extract was hydrolyzed batchwise with Alcalase and Flavourzyme by sequential treatment

(480 min), which was carried out with an initial hydrolysis (180 min) using Alcalase as an endopeptidase and a second one (300 min) using Flavourzyme as an exopeptidase. Hydrolysis parameters for Alcalase were as follows: protein concentration (S) = 2%; enzyme substrate ratio (E/S) = 0.2 AU/g of protein; temperature (T) = 50°C; and pH 8.0. Flavourzyme hydrolysis parameters were: (S) = 2%; E/S = 50 LAPU/g of protein, T = 50°C; and pH 7.0. Samples were withdrawn at certain time intervals and proteases in the aliquots were inactivated by heating at 80°C for 20 min.

The degree of hydrolysis (DH), defined as the percentage of peptide bonds cleaved, was measured by determination of free amino groups by reaction with trinitrobenzenesulphonic acid (Sigma) using leucine as the standard, according to the method of Adler-Nissen [20]. The total number of amino groups was determined by acid hydrolysis with 6 N HCl at 120°C for 24 h.

2.3 Immunodetection assays

2.3.1 Protein electrophoresis and IgE immunoblot experiments

SDS-PAGE was performed according to Laemmli [21]. Samples (20 μg protein *per* lane) were mixed with Laemmli sample buffer (Bio-Rad) and 2-mercaptoethanol (Bio-Rad) heated at 90°C for 10 min, and electrophoresed in 4–20% Tris-HCl linear gradient precast gel (Bio-Rad). Proteins were visualized with Coomassie Brilliant Blue R250 staining. Western blotting was performed by electrophoretic transfer to nitrocellulose membrane (Whatman, Dassel, Germany). After blocking with 3% w/v nonfat milk, 0.1% v/v Tween-20 in PBS (pH 7.4; blocking buffer), the membranes were incubated overnight with the serum pool or individual sera (1:20 dilution), washed, and then treated with mouse anti-human IgE mAb HE-2 ascitic fluid (1:5000 dilution for 1 h) [22]. After washing, a goat anti-mouse IgG peroxidase-conjugated antibody (Pierce, IL, USA) (1:2500 dilution for 1 h) was added. Detection of IgE-binding components was achieved by means of enhanced chemiluminescence, according to the manufacturer's instructions (Amersham, Buckinghamshire, UK). A serum (1:10 dilution) from a patient with a positive specific IgE to *Anisakis* spp. was tested as a negative control.

2.3.2 ELISA

Specific IgE binding to raw lentil extract and hydrolyzed samples was assessed by means of ELISA with the serum pool from the five patients with clinical allergy to lentil. Polystyrene microtiter plates (Costar 3590, Corning, NY, USA) were coated with 100 μL/well of extract (30 μg/mL in PBS) and incubated at 4°C overnight. The wells were washed with 0.01M PBS containing 0.5% Tween-20 v/v and blocked with PBS containing 3% nonfat milk w/v and 0.1% Tween-20 (200 μL/well). Plates were incubated overnight with the serum pool (100 μL/well, 1:20 dilution), and the binding of IgE was detected by incubation for 1 h with mouse anti-human IgE mAb HE-2 ascitic fluid (100 μL/well, 1:5000 dilution) [22] followed by goat anti-mouse IgG peroxidase-conjugated (100 μL/well, 1:2500 dilution for 1 h) (Pierce). Finally, the peroxidase reaction was developed with 50 μL of peroxidase substrate buffer (Dako, Glostrup, Denmark). After 30 min, the reaction was stopped with 50 μL of 4N H_2SO_4, and the OD was measured at 492 nm. The following three negative controls were used: (i) wells coated with non-fat milk (instead of lentil samples) and incubated with the serum pool from the five patients following the process described above; (ii) wells coated with lentil samples and incubated with the negative control serum (instead of the lentil-allergic serum pool) following the process already described; and (iii) wells coated with lentil samples and incubated directly with mouse anti-human IgE and goat anti-mouse IgG.

All the tests were performed in triplicate. The formula: mean [OD] + 3 × SD was calculated for each negative control and the highest value was considered as a cut-off point for positivity.

The percentage of the decrease in antigenic activity was calculated with the formula: $(1-A_H/A_N)$ 100 where A_H is the absorbance value obtained from the hydrolyzed samples and A_N is the absorbance value of the protein extract sample.

3 Results

3.1 Protein extract hydrolysis

Lentil protein hydrolysates were obtained by the sequential action of the endoprotease (Alcalase) (E/S = 0.2 AU/g) and the exoprotease (Flavourzyme) (E/S = 50 LAPU/g). Figure 1 shows the hydrolysis curve of the lentil protein extract using the sequential treatment with Alcalase and Flavourzyme. Protein hydrolysis took place rapidly in the first 30 min; further, progression developed more slowly, reaching 24% DH after 180 min of Alcalase treatment. As hydrolysis of

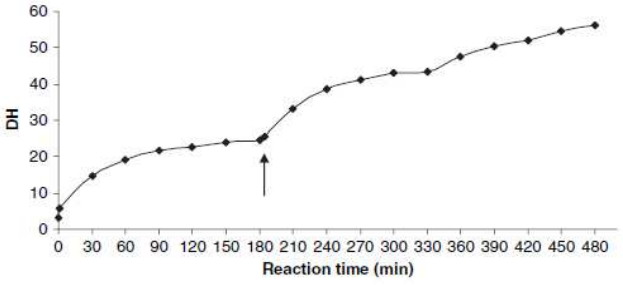


Figure 1. Hydrolysis curves of lentil protein by sequential treatment with Alcalase and Flavourzyme. The arrow indicates the moment Flavourzyme is added. DH: degree of hydrolysis.

protein isolates continued, the DH increased. The combination of both enzymes yielded a DH of 56% at the end of the process (480 min).

3.2 Electrophoretic characterization of protein hydrolysates

Figure 2A shows the SDS-PAGE protein patterns of raw and enzymatically processed lentil extracts. The raw sample showed multiple bands with molecular weights between 14 and 91 kDa. Protein hydrolysates with DH=6% (Alcalase 15 s) showed less stained bands and an increase of low-molecular-weight proteins. Hydrolysates with DH>15% (Alcalase>30 min) did not show any visible electrophoretic bands.

All the samples were analyzed with IgE-immunoblot using the serum pool from the five patients with clinical allergy to lentil (Fig. 2B). Raw lentil showed a complex pattern of IgE-binding proteins in the range of 12–76 kDa. The overall IgE immunoreactivity of lentil was strongly reduced at the end of the sequential endo- and exoprotease hydrolysis (480 min), although bands of 48, 24 and 60 kDa (putative Len c 1, its processing fragments and putative Len c 2, respectively) were still recognized after treatment with Alcalase for 15 s and two tenuous bands of 48 and 60 kDa (putative Len c 1 and Len c 2, respectively) were still present after treatment with Alcalase for 180 min. A spot was recognized after treatment with Flavourzyme during 300 min. This spot could be an artifact since a further immunoblot experiment with raw lentil and with lentil after Flavourzyme treatment for 300 min using the serum pool, showed several IgE-binding proteins in raw lentil and no IgE-binding bands in the hydrolyzed sample (this figure is shown in Supporting Information).

IgE antibody reactivity to raw and hydrolyzed lentil with Alcalase for 180 min and Flavourzyme for 300 min was screened using the five individual sera (Fig. 3). All the sera recognized a wide range of IgE-binding proteins of raw lentil. Four out of five sera recognized fewer IgE-binding proteins combined with a decreased band intensity after Alcalase treatment for 180 min. The IgE reactivity was strongly reduced after Flavourzyme treatment for 300 min. With this treatment, sera from patient 5 did not recognize any proteins; sera from patients 1 and 3 recognized an 18 kDa band (patient 1) and putative Len c 1 (patient 3), both with a decreased intensity. Sera from patients 2 and 4 still detected allergenic proteins of 12 and 14 kDa and a protein between 45 and 48 kDa.

3.3 ELISA

The ELISA test was carried out to assess the reduction of antigenic activity of the protein hydrolysates, using the serum pool from the five patients with clinical allergy to lentil. Specific serum IgE levels > 0.17 OD units (mean [OD] $+3 \times$ SD to raw lentil incubated directly with mouse anti-human IgE and goat anti-mouse IgG: $0.093 + 3 \times 0.024 = 0.17$) were considered as positive. The results are shown in Fig. 4. The IgE reactivity of the hydrolysates was reduced by the sequential action of Alcalase and Flavourzyme. Most proteins from lentil did not maintain their IgE-binding properties during digestion with Alcalase–Flavourzyme. Alcalase was very effective in reducing the antigenic activity of lentil proteins since the enzyme led to a 95% inhibition of antigenicity in the first few seconds (DH 6%); the sequential addition of Flavourzyme caused the destruction of all antigenic epitopes (DH = 56%).

4 Discussion

Enzymatic protein hydrolysates have been reported as a suitable source of protein for human nutrition, as their gastrointestinal absorption seems to be more effective than that of intact proteins [23, 24]. Protein hydrolysates from legumes have been widely used in specific formulations to improve their nutritional and functional properties. These

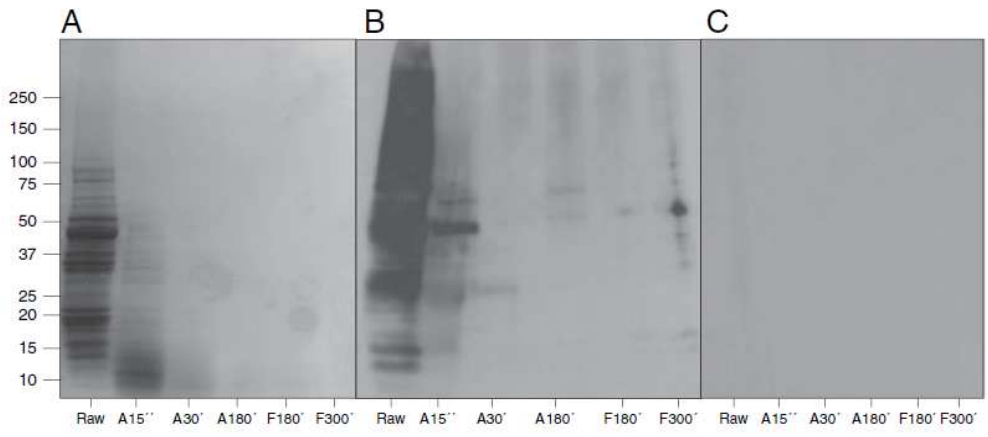


Figure 2. SDS-PAGE (A) and immunodetection with a serum pool from five patients with anaphylactic reaction to lentil (B) or a negative control serum (C) of the following samples: extract from raw lentil (Raw), hydrolysates after sequential treatment with Alcalase during 15 s (A15″), 30 min (A30′), 180 min (A180′), and Flavourzyme treatment during 180 min (F180′) and 300 min (F300′).

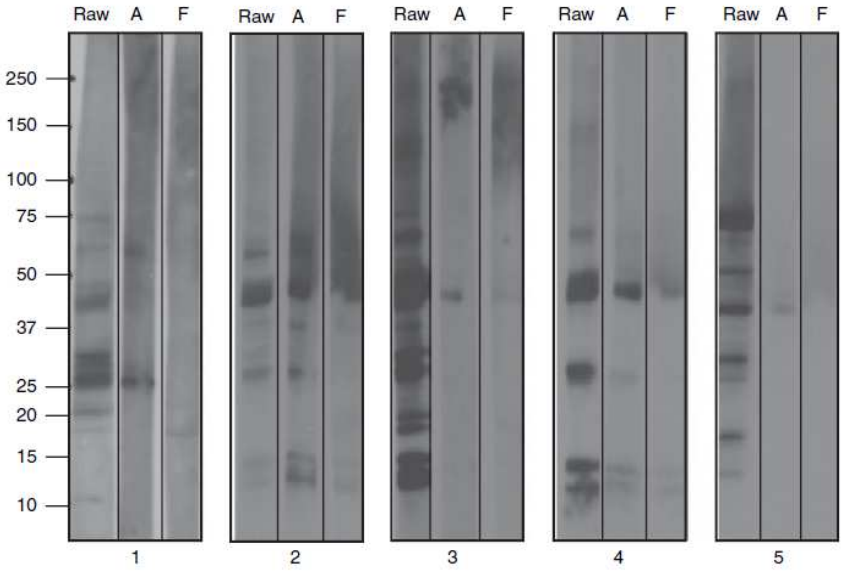
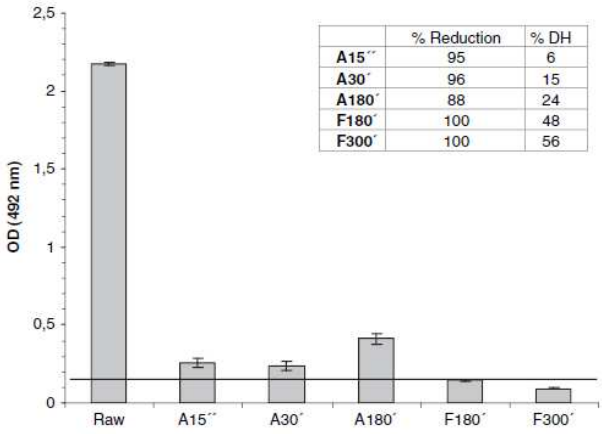


Figure 3. Western blotting of raw lentil and hydrolysates after sequential treatment with Alcalase during 180 min (A) and Flavourzyme during 300 min (F), incubated with individual sera from patients with anaphylactic reaction to lentil.



	% Reduction	% DH
A15´´	95	6
A30´	96	15
A180´	88	24
F180´	100	48
F300´	100	56

Figure 4. Specific IgE to raw lentil and hydrolysates after sequential treatment with Alcalase (15 s, 30 and 180 min) and Flavourzyme (180 and 300 min), using a serum pool from five lentil-allergic patients. Percentages of reduction in antigenic activity and DHs are shown in the table annexed to Fig. 4.

uses include clinical applications, such as geriatric products and therapeutic diets [18].

Food hydrolysate formulae, such as cow's milk or soybean, have been developed with the aim to produce hypoallergenic food with markedly reduced sensitizing capacity. However, residual antigenicity and allergenicity have been reported even in extensively hydrolyzed formulae [25, 26]. Reduction of allergenicity of dietary products may be assessed *in vitro* using various immunological methods. ELISA has been chosen to detect antigenicity in most commercial hypoallergenic hydrolysates because of its sensitivity and relative simplicity [14].

In this study, a sequential hydrolyzation of lentil extract with Alcalase and Flavourzyme was carried out. Clemente *et al.* [14] obtained the most effective reduction of antigenicity in chickpea by a sequential treatment with Alcalase and Flavourzyme compared with both enzymes tested individually. This hydrolysate presented a high protein quality [15].

The present results showed in sequential lentil hydrolysis, a 24% DH at 180 min produced by Alcalase; the addition of Flavourzyme, increased the DH to 56% in the final reaction time (480 min). These data seem to indicate that endoprotease Alcalase produced an initial digestion that increased the number of target sites for the action of the exoprotease Flavourzyme. These values are similar to those obtained for pea [27, 28] and soy [29] protein hydrolysates. Clemente *et al.* [14] obtained a similar DH for chickpea but using also a double *E/S*.

The ELISA results showed that Alcalase elicited a 95% decrease of IgE reactivity before the first minute of hydrolyzation. The immunoblot assay carried out with the serum pool from the five patients with clinical allergy to lentil showed recognition of only three proteins at this time: putative Len c 1 (48 kDa protein), its processing fragment (24 kDa protein), and putative Len c 2 (60 kDa protein); these proteins are probably more resistant to digestion than other immunoreactive proteins. Food allergens are usually resistant to proteases, heat and denaturants allowing them to avoid degradation during food preparation and digestion [30]. The differential thermostability of several lentil IgE-binding proteins recently reported [19] could support the higher resistance to enzymatic digestion of some IgE-binding proteins in comparison with others. After Flavourzyme hydrolyzation, a 100% inhibition was obtained in ELISA and no bands were detected in immunoblotting. However, immunoblot with individual sera showed that two sera detected IgE-binding proteins (12, 14 and 45–48 kDa) in Flavourzyme hydrolyzation during 300 min; the other two

sera exhibited a tenuous recognition of proteins of 18 kDa and putative Len c 1 in the same sample. Sormus de Castro Pinto *et al.* [16] using rabbit antiserum for lentil 11S globulin found weakly immunogenic bands in immunoblotting only after 1 min of treatment of lentil with pepsin and trypsin. The ELISA showed a high reduction of antigenicity with both enzymes. Clemente *et al.* [14] obtained similar results in chickpea, when sequential hydrolysis of Alcalase and Flavourzyme was tested. Humiski and Aluko [27] used Alcalase and Flavourzyme, among other enzymes, to study the effect of proteolytic treatments on pea. They found that both the enzymes produced protein hydrolysates with a significantly higher DH when compared with other proteases (papain, trypsin, and α-chymotrypsin); moreover, the amino acid profile analysis using HPLC system showed that the higher number of peptide fractions obtained for Alcalase and Flavourzyme was probably a reflection of the higher DH of their hydrolysate, which could indicate that more peptide bonds were broken and hence a greater number of peptides were produced.

In conclusion, the results of this study seem to indicate that sequential hydrolysis of lentil with Alcalase and Flavourzyme, produces an important proteolytic destruction of epitopes as shown with *in vitro* assays using sera from patients with clinical allergy to lentil. Although further studies are needed to characterize the biological activity of the residual allergens and to assess the clinical relevance of our findings, this enzymatic procedure seems to be a promising method to obtain hypoallergenic protein hydrolysates. Given the good nutritional value of lentil proteins, this sequential hydrolysis product could be useful to elaborate hypoallergenic food formulae for the population groups with specific nutritional requirements.

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The authors have declared no conflict of interest.

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RESEARCH ARTICLE

Influence of thermal processing on IgE reactivity to lentil and chickpea proteins

Carmen Cuadrado[1], Beatriz Cabanillas[2], Mercedes M. Pedrosa[1], Alejandro Varela[1], Eva Guillamón[1], Mercedes Muzquiz[1], Jesús F. Crespo[2], Julia Rodriguez[2] and Carmen Burbano[1]*

[1] Departamento de Tecnología de Alimentos, SGIT-INIA, Madrid, Spain
[2] Servicio de Alergia, Hospital Universitario 12 de Octubre, Madrid, Spain

In the last years, legume proteins are gaining importance as food ingredients because of their nutraceutical properties. However, legumes are also considered relevant in the development of food allergies through ingestion. Peanuts and soybeans are important food allergens in Western countries, while lentil and chickpea allergy are more relevant in the Mediterranean area. Information about the effects of thermal-processing procedures at various temperatures and conditions is scarce; therefore, the effect of these procedures on legume allergenic properties is not defined so far. The SDS-PAGE and IgE-immunoblotting patterns of chickpeas and lentils were analyzed before and after boiling (up to 60 min) and autoclaving (1.2 and 2.6 atm, up to 30 min). The results indicated that some of these treatments reduce IgE binding to lentil and chickpea, the most important being harsh autoclaving. However, several extremely resistant immunoreactive proteins still remained in these legumes even after this extreme treatment.

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1 Introduction

Legumes are increasingly being regarded as beneficial food ingredients. In fact, they are recommended as staple food by health organizations [1], and dieticians are nowadays tending to encourage their consumption in counseling [2]. Lentils and chickpeas have been reported as a cause of IgE-mediated hypersensitivity reactions, particularly in pediatric patients [3–5]. The frequency of allergy to lentil and chickpea in the Spanish population has been estimated around 20% of children with food allergy [6]. Some subjects allergic to these legumes on ingestion also report symptoms when they inhale vapors from cooking lentils or chickpeas [7–9].

Correspondence: Dr. Carmen Cuadrado, Departamento de Tecnología de Alimentos, SGIT-INIA, Ctra. de La Coruña km 7.5, 28040 Madrid, Spain
E-mail: cuadrado@inia.es
Fax: +34-913572293

Abbreviations: DIC, instantaneous controlled pressure-drop; **PVDF,** polyvinylidene difluoride

Studies investigating the allergenicity of lentils and chickpeas are scarce. So far, no chickpea allergen has been identified but several IgE-binding bands with molecular weights between 10 and 70 kDa have been detected by immunoblotting [5, 9, 10]. A major lentil allergen Len c 1 has been isolated and identified as a 48 kDa vicilin; its processing fragments, corresponding to subunits 12–16 and 26 kDa are also relevant lentil IgE-binding proteins [11, 12].

Heating promotes protein denaturation, aggregation, and structure disruption and therefore has a potential to modify allergenic properties of proteins [13]. The molecular basis of changes in allergenic activity is the inactivation or destruction of epitope structures, the formation of new epitopes, or an enhanced access to cryptic epitopes by denaturation of the native allergen [13]. Plant protein allergenicity may be variably affected by thermal processing, *i.e.* increasing or decreasing IgE immunoreactivity. Thus, the overall effect of this procedure on a complex food allergen cannot be

*Current address: Dr. Eva Guillamón, Centro para la Calidad de los Alimentos, INIA, C/José Tudela prox. 20, Campus Duques de Soria, 42004 Soria, Spain

predicted [14–18]. It has been reported that lentil and chickpea allergens are heat stable after boiling [10, 11, 19]. Our previous studies have observed that boiling in autoclave reduced lupine allergenicity drastically, even abolishing IgE binding [20]. Recently, the high thermal resistance of the major lupine allergens has been established using instantaneous controlled pressure-drop (DIC) treatment, a procedure that combines heat and steam pressure as autoclaving [21].

The aim of this work was to assess changes in the IgE-binding capacity of lentil and chickpea proteins by means of thermal-processing techniques such as boiling and autoclaving.

2 Materials and methods

2.1 Sera

Three different sera groups were employed: (i) 25 individual sera with positive specific IgE to lentil (*Lens culinaris*) (range: 0.53–30.7 kU/L) as quantified by using the CAP-FEIA (fluorescent enzyme immunoassay) system (Pharmacia Diagnostic, Uppsala, Sweden). (ii) Twenty-four individual sera with positive specific IgE to chickpea (*Cicer arietinum*) (range: 0.45–29.6 kU/L). (iii) A serum pool from a different source having specific IgE levels to lentil and chickpea of 31.3 and 29.0 kU/L, respectively. A serum from a patient with specific IgE to *Anisakis* ssp. (9.09 kU/L), specific IgE <0.35 kU/L to lentil and chickpea, and a total serum IgE value of 53.4 kU/L was used as a negative control.

2.2 Plant material, heat treatments, and protein extracts

Mature lentil seeds (*L. culinaris* var. Guareña) obtained from the Servicio de Investigación y Tecnología Agraria (Valladolid, Spain) and mature chickpea seeds (*C. arietinum* var. Athenas) obtained from the IFAPA (Córdoba, Spain) were subject to different treatments, including boiling and autoclaving. Seeds (1:10 w/v) were boiled in water at 100°C for 15, 30, and 60 min. Using a table top autoclave (CertoClav model IPX4, Austria), legume seeds (1:5 w/v) were treated at 1.18 and 2.56 atm for 5, 15, and 30 min in the case of lentils and 15 and 30 min in the case of chickpeas.

Raw and thermal-processed seeds were milled to pass through a 1 mm sieve (Tecator, Cyclotec 1093, Sweden) and the resulting meal was defatted with *n*-hexane (34 mL/g of flour) for 4 h with agitation and air-dried after filtration of the *n*-hexane. Defatted flour was extracted with 0.1 M PBS buffer pH 7.4 plus 0.15 M NaCl at a 1:10 w/v ratio during 1 h at 4°C with stirring. The extract was clarified by centrifugation at $27\,000 \times g$ for 30 min at 4°C, and the supernatants were dialyzed against distilled H_2O during 24 h at 4°C using dialysis membrane (Spectra/Por, Serva, Heidelberg, Germany) with a cut-off of 3.5 kDa and then freeze-dried. The residue obtained after extraction and the cooking water were freeze-dried for analysis. The soluble protein content of each sample was measured by the Bradford dye-binding assay (Bio-Rad, Hercules, CA, USA) using BSA (Sigma, St. Louis, MO, USA) as a standard. The total N was determined using the Kjeldahl procedure [22]. The total crude protein content was calculated as $N \times 5.45$ [23].

2.3 Protein electrophoresis and immunoblotting

Denaturing protein electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [24]. Samples were mixed with XT Sample Loading Buffer (Bio-Rad) and XT Reducing Agent (Bio-Rad), heated to 90°C for 10 min, and electrophoresed in 12% Bis-Tris Criterion XT Precast Gel (Bio-Rad). Proteins (16 μg protein/lane) were visualized with CBB R250 staining. IgE immunoblotting analysis was performed by electrophoretic transfer to polyvinylidene difluoride (PVDF) at 250 mA during 100 min at room temperature [25]. After blocking with 5% BSA w/v in PBS buffer, membranes were incubated overnight with the serum pool or individual sera (1:10 dilution), washed, and then treated with mouse anti-human IgE mAb HE-2 ascitic fluid (1:3000 dilution for 2 h) [26]. After washing, a rabbit anti-mouse IgG peroxidase-conjugated antibody (1:5000 dilution for 1 h; DAKO, Glostrup, Denmark) was added. Detection of IgE-binding components was achieved by means of enhanced chemiluminescence, according to the manufacturer's instructions (Amersham Biosciences, Little Chalfont, UK).

Coomassie-stained gels and membranes were scanned and the molecular weight of the bands was assessed using Quantity One software (Bio-Rad) and the low-range SDS-PAGE protein mixture (Sigma) as standard.

2.4 Skin prick tests

Skin testing was carried out after *in vitro* experiments were completed. For this purpose, 12 additional patients sensitized to legumes were asked to undergo skin testing with raw and thermal-processed extracts. After obtaining the informed consent, approved by the Ethic Committee (Permission No. 0312150129) skin prick tests (1:10 w/v) were performed in duplicate on the volar side of the forearm according to standard methods [27]. A mean wheal diameter of 3 mm or greater (15 min after puncture) compared with that produced by the negative control was considered a positive response. Positive and negative controls for skin testing were histamine dihydrochloride (10 mg/mL) and saline solutions, respectively.

3 Results

Thermal processing can make that some seed proteins be un-extractable and left in the residue and some dissolve in the cooking medium. Therefore, the protein content, SDS-PAGE, and IgE-immunoblotting have been analyzed in defatted legume flour, extract, residue, and cooking water of lentil and chickpea before and after heat treatments (data not shown). The results supported that more protein remained in the residue as un-extractable material upon processing than in raw samples and part of extractable protein were solubilized into the cooking water. However, there was not substantially different IgE-immunoreactivity profile among these fractions (flour, extract, residue, and water); therefore, the IgE-binding capacity of the PBS extract could be considered as representative of the overall immunoreactivity of these foods.

3.1 Effects of heating on lentil allergens

Figure 1 shows the SDS-PAGE protein patterns of raw and thermal-processed lentil extracts. All these extracts were analyzed by IgE-immunoblot using the serum pool (Fig. 2). SDS-PAGE and IgE-immunoblot band patterns are similar in raw and 15-min boiled lentil showing multiple allergenic proteins. After boiling for 30 min, a putative Len c 1, the major lentil allergen, is eliminated, but multiple immunoreactive proteins are still present. The autoclave process at harsh conditions (2.6 atm) induces major changes in lentil immunoreactivity (lanes 8–10). The number and intensity of bands are reduced after 15 min autoclaving (2.6 atm) with several heat-stable allergenic proteins still being present. A few extremely resistant immunoreactive bands (16, 18, 21, 30, and 43 kDa) are detected at the highest pressure and treatment time applied (lane 10).

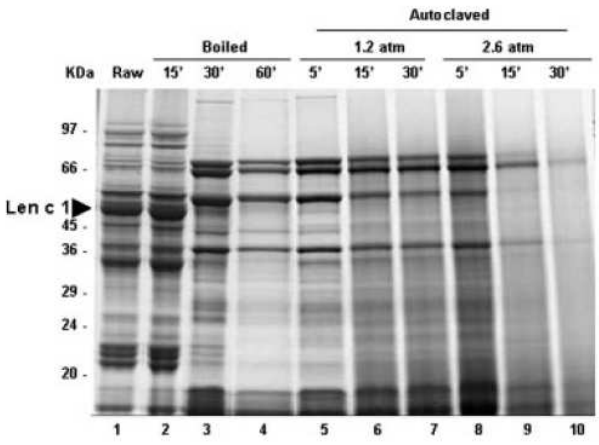

Figure 1. SDS-PAGE patterns of protein extract from raw (lane 1) and processed lentil (lanes 2–10) samples. Arrow indicates the position of the major lentil allergen.

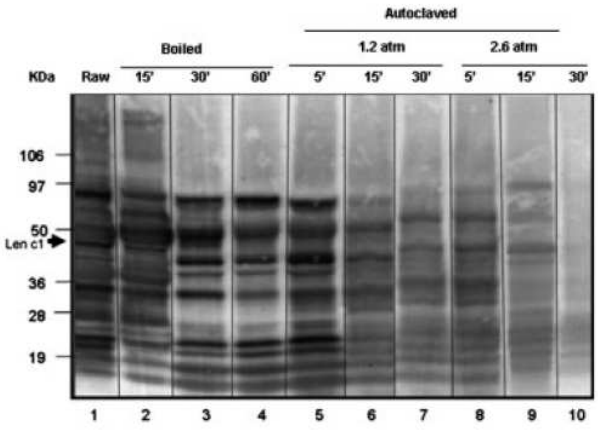

Figure 2. IgE immunoblot analysis of raw (lane 1) and processed lentil (lanes 2–10) with a serum pool from subjects with specific IgE to lentil: 31.3 kU/L. Arrow indicates the position of the major lentil allergen.

IgE antibody reactivity to 60-min boiled and autoclaved lentil (2.6 atm for 30 min) was further screened using the 25 individual sera (Fig. 3A and B). Boiled lentils had a complex pattern of IgE-binding proteins in the range of 5–107 kDa. The proteins recognized with higher frequency by the sera were: 37 kDa (32%); 50 kDa (32%); and the 45, 48, and 51 kDa bands that are detected by 25% of the sera.

The overall immunoreactivity of lentils was strongly reduced after autoclaving at 2.6 atm for 30 min, although 11 out of 25 tested sera (44%) reacted to immunoreactive proteins, 19 and 23 kDa being the allergens most frequently recognized. Other heat-resistant allergenic proteins (16, 17, 26, 35, 36, 39, 45, and 50 kDa) were still detected by 2 out of 25 sera. Autoclaving lentils at 2.6 atm for 30 min abolished IgE antibody reactivity from these sera to previously detected components such as 48, 37, and 51 kDa.

Table 1 depicts results of skin prick test with raw and thermal-processed extracts (60-min boiled and autoclaved at 2.6 atm for 30 min) of lentils and chickpeas in 12 patients sensitized to legumes. Six patients showed a positive result to raw lentil, but only two patients (no. 5 and 12) reacted to boiled and autoclaved lentil. Patient no. 5 showed higher skin reactivity to boiled lentil in comparison with raw.

3.2 Effects of heating on chickpea allergens

The SDS-PAGE patterns of the protein extracts of raw, boiled, and autoclaved chickpea seeds are shown in Fig. 4. Figure 5 shows the IgE immunoblotting analysis of chickpea extracts using the serum pool. Raw and boiled chickpea up to 30 min have similar SDS (Fig. 4) and IgE-immunoreactive band pattern (Fig. 5). A decrease in the number and intensity of bands is observed after boiling for 60 and 15 min autoclaving (1.2 atm) with several allergenic proteins still

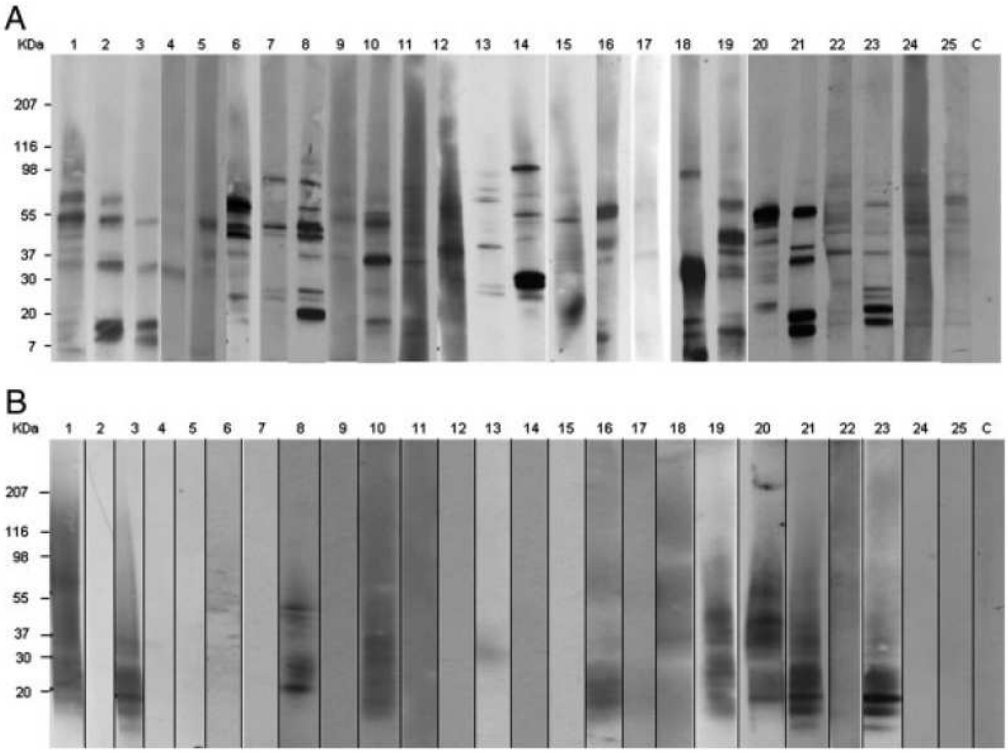

Figure 3. IgE antibody reactivity by immunoblotting to allergens of 60-min boiled (A) and autoclaved lentil (2.6 atm for 30 min) (B). Lentil proteins were resolved by means of SDS-PAGE and transferred to PVDF membranes. All 5-mm strips from each blot were tested for reactivity to serum IgE from 25 subjects sensitized to lentil-specific IgE. Lanes 1–25 represent sera from subjects 1–25, respectively. Lane C represents a negative control serum.

Table 1. Results of skin prick testing with raw and thermal-processed extracts (60-min boiled and autoclaved at 2.6 atm for 30 min) of lentils and chickpeas in patients sensitized to legumes

Patient no.	Specific IgE (kU/L)					SPT (mm) lentil			SPT (mm) chickpea		
	Lentil	Chickpea	Peanut	Soybean	Green bean	Raw	Boiled	Autoclaved	Raw	Boiled	Autoclaved
1	–	0.70	–	–	–	0	0	0	0	0	0
2	–	–	1.70	–	–	0	0	0	0	0	0
3	–	–	0.87	–	–	0	0	0	0	0	0
4	6.13	28.6	4.55	11.8	–	0	0	0	0	0	0
5	0.34	0.48	4.38	2.09	–	3	9.25	3.25	4.75	5.75	6.5
6	0.43	0.12	1.26	–	–	4	0	0	0	0	0
7	0.94	–	1.01	0.62	–	5.75	0	0	0	0	0
8	0	0	–	–	0.35	0	0	0	3.75	0	0
9	1.85	1.30	0.88	0.42	–	6.5	0	0	8	0	0
10	0.62	–	–	–	–	0	0	0	0	0	0
11	0.36	–	1.23	–	–	4.75	0	0	0	0	0
12	7.78	4.60	–	4.22	3.68	6.75	7	6	0	7.25	6.5

visible. Immunoreactivity is strongly reduced after autoclaving and the reduction is higher as pressure and treatment time increase. At 2.6 atm (15 min) four heat-stable-immunoreactive bands are identified (40, 26, 19, and 16 kDa) and after 30 min, two bands (19 and 16 kDa) are still detected.

IgE immunoblots of chickpea, boiled for 60 min and autoclaved at 2.6 atm for 30 min with the individual sera of 24 patients, are shown in Fig. 6A and B. Multiple immunoreactive proteins are identified in boiled chickpeas in the range of 5–124 kDa. The 25 and 42 kDa proteins are recognized by approximately 33% (5 out of 24 sera) of the sera. Other bands are also detected by approximately 21% (8 out of 24 sera) of the sera (21, 23, 27, 31, and 37 kDa).

After autoclaving at 2.6 atm for 30 min, several immunoreactive chickpea proteins are still recognized by 10 out of the 24 sera. The 11 and 28 kDa allergens show the highest recognition frequency (3 sera). The rest of the immunoreactive bands of boiled chickpea extract were eliminated by autoclaving.

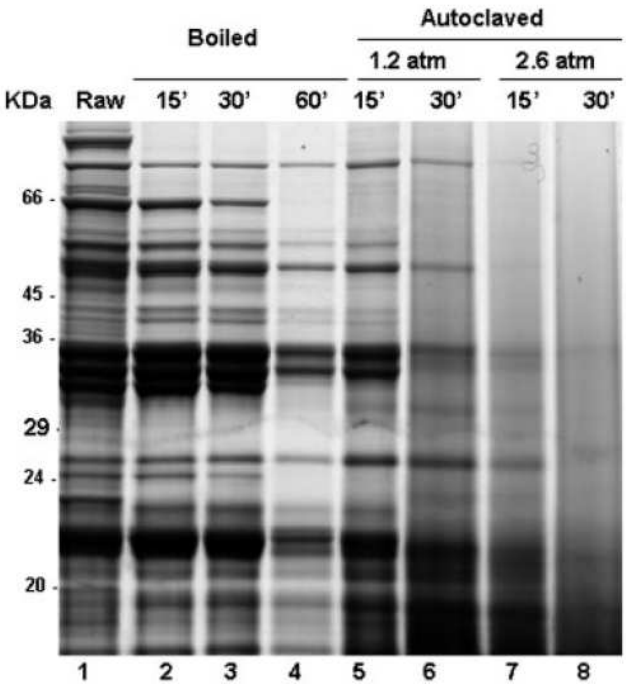

Figure 4. SDS-PAGE patterns of protein extract from raw (lane 1) and processed chickpea (lanes 2–8) samples.

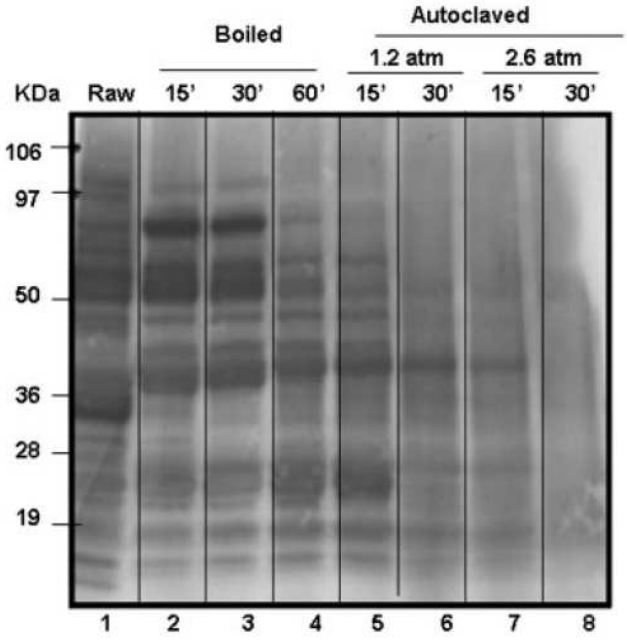

Figure 5. IgE immunoblot analysis of raw (lane 1) and processed chickpea (lanes 2–8) with a serum pool from subjects with specific IgE to chickpea: 29.0 kU/L.

Skin testing with raw extract elicited a positive response in 3 out of 12 patients, whereas boiled and autoclaved extracts elicited positive reactions in two patients (Table 1). Patient no. 12 showed a negative skin prick result to raw chickpea, while reacting to boiled and autoclaved extracts.

4 Discussion

Thermal and nonthermal processing can increase or decrease allergenicity of food proteins or result in new allergens (neoallergens) being formed. Therefore, the study of food processing seems to be important to evaluate food allergenicity [28]. Most investigations dealing with the influence of thermal processing on legume allergenicity have been focused on peanut and soybean [14, 29–32]. Several authors have described an increase in peanut allergenicity with roasting procedures [14, 15, 33, 34]. Beyer *et al.* [14] observed that in contrast to roasting, frying and boiling peanuts reduced IgE-binding reactivity to the major peanut allergens Ara h 1, Ara h 2, and Ara h 3. Burks *et al.* [29] did not find a significant decrease of IgE binding after heating (100°C, up to 60 min) soybean protein extracts. However, boiling for 120 min and microwave heating of soybean seems to decrease allergenicity, as only half of soybean-allergic patients were shown to have detectable specific IgE against heated soybean protein [35].

The present results demonstrated that boiling treatments produce little modifications on SDS-PAGE pattern (Figs. 1 and 4) and IgE-binding capacity (Figs. 2 and 5) of lentil and chickpea allergenic proteins. In fact, the presence of multiple IgE-binding proteins when 60-min boiled lentil and chickpea extracts were tested with individual sera (Figs. 3A and 6A) demonstrate that both lentil and chickpea still contain a relevant number of heat-stable-immunoreactive proteins.

To date, there are few studies describing the effect of heat treatment on lentil and chickpea allergenicity. Boiling of lentil seeds did not reduce immunoreactivity as demonstrated by ELISA inhibition experiments and immunoblotting [11, 19]. Martinez *et al.* [10] analyzed the allergenic composition of raw and boiled chickpea extract detecting similar IgE-binding proteins in both. Our results confirmed the thermo stability of lentil and chickpea allergens.

The present findings suggest that autoclaving produces a severe effect on the integrity and structure of lentil and chickpea proteins, which runs in parallel with a decrease in IgE-binding properties of these legume seeds. To date, little is known about the effect of the pressure technique process, *i.e.* autoclaving on allergenic proteins. Venkatachalam *et al.* [16] and Brenna *et al.* [17] demonstrated that the allergenicity of almond proteins and peach nectar (Pru p 1) protein is maintained after autoclaving at 1.2 atm (121°C) during 30 min while allergenicity of green pea is reduced after autoclaving at 121°C for 15 min [18]. In our previous studies lupine allergenicity could be abolished by autoclaving at 2.6 atm for 30 min or by DIC at 6 bar (5.9 atm) during 3 min, whereas it was only slightly affected by boiling, microwave, and extrusion-cooking, demonstrating the high thermal resistance of the major lupine allergens [20, 21].

From our point of view, aggregation and differential solubility cannot totally explain the large amount of modifications of allergenic proteins observed upon processing, especially autoclaving, in contrast to what reported by Mondoulet *et al.* [15]. Our data are more in agreement with an extensive

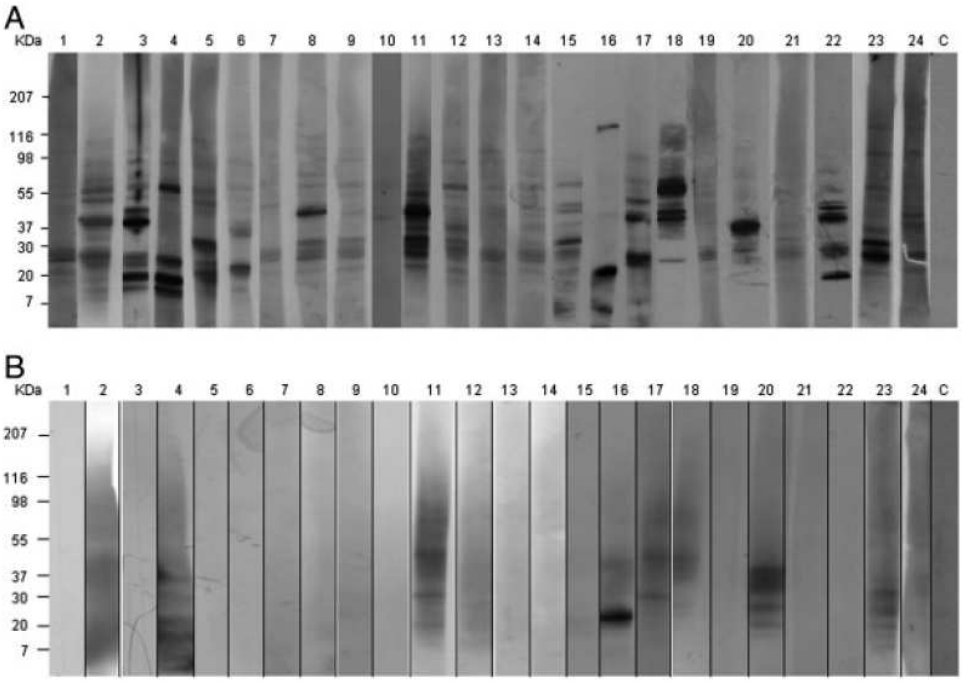

Figure 6. IgE antibody reactivity by immunoblotting to allergens of 60-min boiled (A) and autoclaved chickpea (2.6 atm for 30 min) (B). Chickpea proteins were resolved by means of SDS-PAGE and transferred to PVDF membranes. All 5-mm strips from each blot were tested for reactivity to serum IgE from 24 subjects sensitized to chickpea specific IgE. Lanes 1–24 represent sera from subjects 1–24, respectively. Lane C represents a negative control serum.

degradation of the proteins that would produce short peptides situated in the low MW portion of the gels, causing the smear observed in the low-MW portions of electrophoretic lanes of autoclaved samples (Figs. 1, 2, 4, and 6). This could explain the disappearance of many IgE-immunoreactive band proteins [28]. We observed that the IgE immunoreactivity of lentil and chickpea extracts only decreased significantly at extreme conditions, when the maximum pressure and time treatment (autoclaving at 2.6 atm during 30 min) were applied. When autoclaved, lentil samples at harsh conditions were analyzed by immunoblot with individual sera or a serum pool, the putative lentil major allergen Len c 1 could not be detected, although some extremely resistant bands still conserved the IgE-binding capacity (Figs. 2 and 3). The number of sera reacting to IgE-reactive proteins in autoclaved lentil extract is higher in comparison with autoclaved chickpea, a fact that suggests that the thermal stability of lentil allergens is stronger than that of chickpea allergens.

In summary, we can conclude that autoclaving at 2.56 atm for 30 min produces a significant decrease of IgE-binding capacity of lentil and chickpea allergens, as less than half of individual sera and patients react against these extremely treated legume seeds. However, several immunoreactive proteins still remained in these legumes upon harsh autoclaving. Further work on their identification would need to be undertaken.

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The authors have declared no conflict of interest.

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Heat and pressure treatments effects on peanut allergenicity

Beatriz Cabanillas [a,*,1], Soheila J. Maleki [b,1], Julia Rodríguez [a], Carmen Burbano [c], Mercedes Muzquiz [c], María Aránzazu Jiménez [a], Mercedes M. Pedrosa [c], Carmen Cuadrado [c], Jesús F. Crespo [a]

[a] Servicio de Alergia, Hospital Universitario 12 de Octubre. Instituto de Investigación Hospital 12 de Octubre (i+12), Avenida de Córdoba s/n, 28041 Madrid, Spain
[b] US Department of Agriculture, Agriculture Research Service, Southern Regional Research Center, 1100 Robert E. Lee Boulevard, New Orleans, LA, United States
[c] Departamento de Tecnología de Alimentos, SGIT-INIA, Ctra. de La Coruña km 7.5, 28040 Madrid, Spain

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ABSTRACT

Peanut allergy is recognized as one of the most severe food allergies. The aim of this study was to investigate the changes in IgE binding capacity of peanut proteins produced by thermal-processing methods, including autoclaving. Immunoreactivity to raw and thermally processed peanut extracts was evaluated by IgE immunoblot and skin prick test in patients with clinical allergy to peanut. Roasted peanut and autoclaved roasted peanut were selected for IgE ELISA experiments with individual sera, immunoblot experiments with antibodies against peanut allergens (Ara h 1, Ara h 2 and Ara h 3), digestion experiments, and circular dichroism spectroscopy. *In vitro* and *in vivo* experiments showed IgE immunoreactivity of roasted peanut proteins decreased significantly at extreme conditions of autoclaving. Circular dichroism experiments showed unfolding of proteins in autoclave treated samples, which makes them more susceptible to digestion. Autoclaving at 2.56 atm, for 30 min, produces a significant decrease of IgE-binding capacity of peanut allergens.

1. Introduction

Peanut allergy is one of the most severe food allergies due to its life-threatening nature and persistency (Sicherer & Sampson, 2007). Peanut allergy prevalence seems to have increased in the western world during the past decades. An estimate of the prevalence of peanut allergy in US children was 1.4% in 2008 compared with 0.8% in 2002 and 0.4% in 1997 in a self-reported population survey (Sicherer, Muñoz-Furlong, Godbold, & Sampson, 2010).

Foods, including peanuts, are subjected to different processing methods to improve their quality, preservation, safety, suitability for specific product applications, etc. (Sathe & Sharma, 2009). The types of modifications that food proteins may undergo during processing includes protein unfolding and aggregation and chemical modification (Mills, Sancho, Rigby, Jenkins, & Mackie, 2009). These modifications could alter food allergenicity, i.e. increasing or decreasing IgE reactivity (Cabanillas-Martín, Crespo, Burbano, & Rodríguez, 2010; Cuadrado et al., 2009). Therefore, understanding food processing seems to be important in food allergenicity.

It has been recognized that roasted peanuts are more allergenic than raw peanuts (Beyer et al., 2001; Chung & Champagne, 1999, 2001; Kopper et al., 2005; Maleki, Chung, Champagne, & Raufman, 2000; Maleki et al., 2003). Roasted peanut extracts bind IgE from patients with peanut allergy at approximately 90-fold higher levels than raw peanuts, and the protein modifications induced by Maillard reaction contribute to the observed effect (Chung & Champagne, 1999, 2001; Maleki et al., 2000). Previous studies have demonstrated that autoclaving at 2.56 atmospheres (atm) for 30 min (min) and "instantaneous controlled pressure drop (DIC)" at 6 bar (5.9 atm) during 3 min reduced IgE-binding capacity of lupine allergens, whereas it was only slightly affected by boiling, microwave and extrusion-cooking, demonstrating the high thermal resistance of the major lupine allergens (Alvarez-Alvarez et al., 2005; Guillamón et al., 2008).

Autoclaving at 2.56 atm, for 30 min, produced a relevant decrease in the IgE-binding capacity of lentil and chickpea allergens. However, several immunoreactive proteins still remained in these legumes upon harsh autoclaving (Cuadrado et al., 2009).

We sought to investigate the changes in IgE binding capacity of peanut proteins produced by thermal-processing methods, including autoclaving.

2. Materials and methods

2.1. Patients and sera

Serum samples from 54 patients sensitized to peanut were used in this study. Nineteen of them (Nos. 1–19) had a clinical history of peanut allergy, confirmed on the basis of either a convincing history of severe systemic anaphylaxis after peanut ingestion or a

* Corresponding author. Tel.: +34 913908660; fax: +34 913908261.
E-mail address: bcabanillas.hdoc@salud.madrid.org (B. Cabanillas).
[1] These authors contributed equally to this study.

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positive double-blind, placebo-controlled food challenge (DBPCFC) with peanut, a positive skin prick test response and a specific IgE level to peanut, greater than 0.35 kU/l (median = 2.79 kU/L; range, 0.47→100 kU/L), quantified with the CAP-FEIA System (Phadia, Uppsala, Sweden). The other 35 patients (Nos. 20–54) were sensitized to peanut, having a specific IgE level to peanut, greater than 0.35 kU/l (median = 5.44 kU/l; range, 0.36→100 kU/L). A serum pool from 4 out of 19 patients with actual peanut allergy (Nos. 5, 8, 9 and 11) and individual sera from all patients were used in IgE immunodetection assays. A serum pool from three patients with specific IgE to *Anisakis spp.* was used as a negative control.

After informed consent, seven additional patients with confirmed clinical allergy to peanut underwent skin testing with raw and thermally processed peanut extracts. Two healthy subjects were tested with the same protein extracts as a control group. The study was approved by the Ethics Committee of the Hospital Universitario 12 de Octubre of Madrid (Permission No. 0312150129).

2.2. Plant material, heat treatments and protein extracts

Raw, fried and roasted peanuts (Virginia variety) obtained from Aperitivos Medina SL (Spain) were used in the study. Raw peanut seeds (1:10 w/v) were boiled in water, at 100 °C, for 60 min. Roasted peanuts seeds were autoclaved using a tabletop autoclave (CertoClav Multicontrol IPX4, Traun, Austria) at 121 °C (1.18 atm) for 15 and 30 min and at 138 °C (2.56 atm) for 15 and 30 min.

Raw and thermally processed peanut seeds were milled to pass through a 1 mm sieve (Tecator, Cylotec 1093, Höganäs, Sweden) and the resulting meal was defatted with *n*-hexane (34 ml/g of flour) for 4 h, shaken, and air-dried after filtration of the *n*-hexane. Defatted flour was extracted twice in a solution of 50 mM Tris–HCl, pH 8.0, plus 500 mM NaCl at a 1:10 w/v ratio for 1 h, at 4 °C, by stirring. After centrifugation (27000*g*, for 20 min, at 4 °C) the supernatants were dialyzed against H_2O (cut-off point, 3.5 kDa), for 48 h at 4 °C and freeze-dried. The protein content of each sample was measured according to the Bradford dye-binding assay (Bio-Rad, Hercules, CA) using BSA (Sigma, St. Louis, MO) as a standard.

2.3. Immunodetection assays

2.3.1. Protein electrophoresis and IgE immunoblot experiments

SDS–PAGE was performed according to Laemmli (1970). Samples (10 μg per well) were mixed with Laemmli sample buffer (Bio-Rad) and 2-mercaptoethanol (Bio-Rad) heated at 90 °C for 10 min, electrophoresed in 4–20% Tris–HCl linear gradient precast gel (Bio-Rad). Proteins were visualized with Coomassie brilliant blue R250 staining. Western blotting was performed by electrophoretic transfer to polyvinylidene difluoride (PVDF) membranes at 250 mA for 100 min, at room temperature, essentially according to the method of Towbin, Staehelin, and Gordon (1979). After blocking with 5% BSA (w/v) in PBS, membranes were incubated overnight with the serum pool from patients with clinical allergy to peanut (Nos. 5, 8, 9 and 11, 1:5 dilution) or individual sera (1:10 dilution) washed, and then treated with mouse anti-human IgE mAb HE-2 ascitic fluid (1:3000 dilution for 2 h) (Sanchez-Madrid, Morago, Corbi, & Carreira, 1984). After washing, a rabbit anti-mouse IgG peroxidase-conjugated antibody (1:5000 dilution for 1 h; DAKO, Glostrup, Denmark) was added. Detection of IgE-binding components was achieved by means of enhanced chemiluminescence, according to the manufacturer's instructions (Amersham Biosciences, Little Chalfont, United Kingdom). A serum pool (1:10 dilution) from three patients with specific IgE to *Anisakis spp.* was tested as a negative control.

2.3.2. ELISA

Specific IgE binding was determined by means of indirect ELISA in 14 individual sera from patients with clinical allergy to peanut (Nos. 2, 4, 6, 7, 9, 10, 12, 15, 17) and sera from patients sensitized to peanut (Nos. 21, 22, 24, 32, 33). The selection of these specific patients was made attending serum availability. Polystyrene 96-well microtiter plates (Costar 3590, Corning) were coated with 100 μl of extract at 30 μg/ml in PBS and incubated at 4 °C overnight. Wells were washed with PBS and 0.5% Tween 20 (v/v) and blocked with PBS containing 3% nonfat milk (w/v) and 0.1% Tween 20. Plates were incubated with individual sera (1:2 dilution) and binding of IgE was detected by mouse anti-human IgE mAb HE-2 ascitic fluid (1:5000 dilution for 1 h) (Sanchez-Madrid et al., 1984) followed by goat anti-mouse IgG peroxidise-conjugated (1:2500 dilution for 1 h, Pierce Chemical Co, Rockford, Ill). The peroxidase reaction was developed with 50 μl of peroxidase substrate buffer (Dako). After 30 min, the reaction was stopped with 50 μl of 4 N H_2SO_4, and the optical density (OD) was measured at 492 nm. All the tests were performed in triplicate.

2.3.3. Anti Ara h 1, Ara h 2 and Ara h 3 immunoblots

For anti Ara h 1, Ara h 2 and Ara h 3 experiments, defatted flours were solubilized with extraction buffer (50 mM Tris, 500 mM NaCl), followed by sonication and centrifugation at 5500*g*, for 15 min. The supernatant was removed and saved (soluble fraction). The pelleted fractions, after centrifugation (insoluble fraction), were solubilized by boiling for 5 min in standard electrophoresis sample buffer, containing 2% sodium dodecyl sulfate (SDS) and reducing agent (Invitrogen). The samples in SDS-sample buffer were centrifuged at 5500*g* for 15 min. The supernatants were removed, aliquoted and stored at −20 °C.

The proteins in the soluble and insoluble fractions were electrophoresed and transferred to PVDF membranes. Blocking was carried out for 1 h, at room temperature, in 5% blotto. Chicken anti-raw Ara h 1 (1:10000), chicken anti-raw Ara h 2 (1:8000) and chicken anti-raw Ara h 3 (1:5000) (custom synthesized by Sigma Immunosys, The Woodlands, TX) were diluted in 5% blotto and incubated with the PVDF membrane for 1 h, at room temperature. These antibodies have been shown to recognize both raw and thermally processed forms of these proteins (Schmitt, Nesbit, Hurlburt, Cheng, & Maleki, 2010). The HRP-labeled-anti-chicken IgY (1:100,000) (Sigma Immunosys, The Woodlands, TX), was diluted in 2% blotto and incubated with membranes for 30 min at room temperature. Detection was achieved as described above.

2.4. Digestion reactions with trypsin

Roasted peanut and autoclaved roasted peanut were selected for digestion experiments with trypsin. Soluble fractions were incubated in the presence of 0.0168 μM trypsin in a solution of 50 mM Tris, 1 mM EDTA at pH 8.4, at 37 °C. Aliquots were taken for SDS–PAGE analysis at 1, 3, 5, 10, 30 min, 1 h, 2 h, and overnight (~15 h). The gels were stained for 1 h using GelCode Blue Stain Reagent according to manufacturer's instructions (Pierce, Rockford, IL).

2.5. Circular dichroism (CD) spectroscopy

Far UV (185–250 nm) circular dichroism spectra of roasted peanut and autoclaved roasted peanut (1.18 atm and 2.56 atm, 15 and 30 min) were obtained. Samples were desalted through use of disposable gel filtration columns (Bio-Rad, Hercules, CA) into Milli-Q water and immediately used in CD measurements. Protein concentration was 0.1 mg/ml and spectra were obtained at room temperature with a JASCO 815 spectropolarimeter equipped with a Peltier temperature control system (Japan Spectroscopic Co. Ltd., Tokyo,

Japan). Average of two measurements was obtained using Jasco CD Manager Software. Data was analyzed with CDPro.

2.6. Skin prick tests

Skin prick tests were performed through a standard technique with sterile needles (Alk-Abelló, Hørsholm, Denmark). Protein extracts of raw, boiled, fried, roasted and autoclaved roasted peanut (2.56 atm, 30 min) (10 mg/ml protein in PBS); a positive control (histamine dihydrochloride, 10 mg/ml); and a negative control (PBS) were applied on the volar side of the forearm in duplicate. The mean diameters of the wheal were measured after 15 min. To be considered positive, the wheal had to be at least 3 mm greater than that elicited by the negative control.

3. Results

3.1. Electrophoretic characterization

The SDS–PAGE patterns of the protein extracts of raw, boiled, fried, roasted and autoclaved roasted peanut seeds are presented in Fig. 1A1. Similar patterns were observed in raw, boiled, fried and roasted peanut extracts. Roasted peanut after autoclaving (1.18 and 2.56 atm) showed less stained bands but an increase of low molecular weight smear. Western blotting was performed using a serum pool from four patients with actual peanut allergy (Nos. 5, 8, 9 and 11) (Fig. 1A2). Similar allergenic proteins were present in raw, fried and roasted peanut extracts with the exception of a high molecular weight protein (121 kDa) that is significantly reduced in fried peanut extract (Fig. 1A2). The IgE-binding pattern of boiled peanut extract showed differences compared to raw, roasted and fried peanut extract: the 65 kDa band (Ara h 1) and Ara h 2 (17–18 kDa) were less visible in this extract. After autoclaving of roasted peanut, a reduction in the total IgE-reactive bands was seen (Fig. 1A2). Bands of 121, 65, 37 kDa and two low molecular weight proteins (17, 14 kDa) were not bound by IgE in any autoclaved treatment (1.18 or 2.56 atm). Two immunoreactive bands of 26 and 33 kDa were still detected at high pressure (2.56 atm) applied for 15 min, however under the same pressure conditions applied during 30 min, none of them were recognized.

Western blotting using a serum pool from three patients with specific IgE to *Anisakis spp.* (negative control; Fig. 1A3) did not show any reactive bands.

IgE antibody reactivity to roasted peanut extract and autoclaved roasted peanut extract under extreme conditions (2.56 atm, 138 °C, 30 min) was further screened using fifty-four individual sera from patients sensitized to peanut. Western blotting of roasted (B1) and autoclaved roasted peanut extracts (B2) are shown in Fig. 1B. A complex pattern of bands from 13 to 121 kDa was detected in roasted peanut, with proteins of 65, 26, 22, 20 and 17 kDa recognized by 90%, 70%, 70%, 67.1% and 60% respectively. Twelve out of 54 (22%) sera recognized some proteins in autoclaved roasted peanut at 2.56 atm for 30 min.

3.2. ELISA

ELISA assay using roasted peanut and autoclaved roasted peanut (2.56 atm, 138 °C, 30 min) as a solid phase led to determine the specific IgE levels in the individual sera from fourteen peanut-sensitized patients. The percentage of the decrease in antigenic activity was calculated with the formula: $(1 - A_H/A_N)\,100$ where A_H is the absorbance value obtained from autoclaved roasted peanut

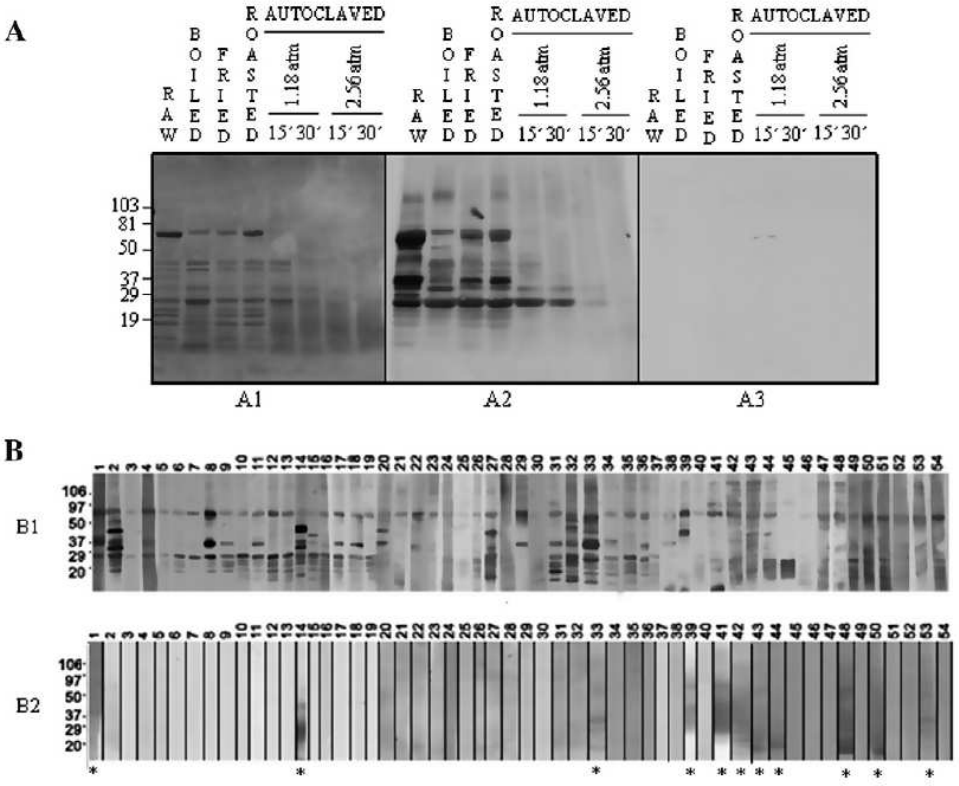
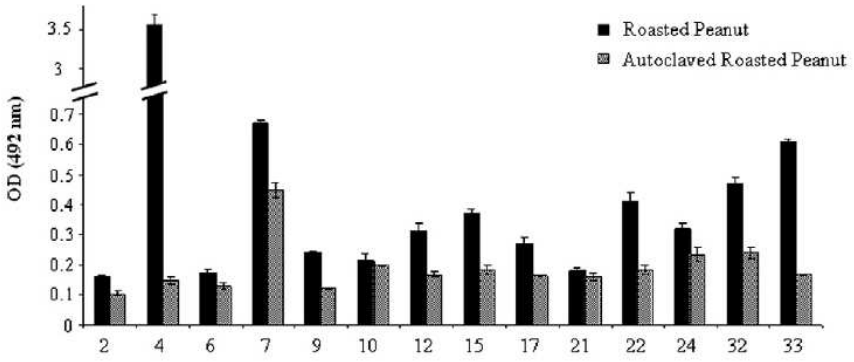


Fig. 1. A. SDS–PAGE (A1) and western blot (A2 and A3) of raw, boiled, fried, roasted peanut and autoclaved roasted peanut. Western blot A2 was carried out using a serum pool from 4 patients with actual peanut allergy (Nos. 5, 8, 9 and 11). Western blot A3 was carried out using a serum pool from 3 patients with allergy to *Anisakis* spp. 1B. Western blot of roasted peanut (B1) and autoclaved roasted peanut at 2.56 atm, 30 min (B2) incubated with sera from patients with actual peanut allergy (lanes 1–19) and sera from patients sensitized to peanut (20–54). Sera recognizing some proteins in autoclaved roasted peanut at 2.56 atmospheres for 30 min are marked with an asterisk.



Serum number	2	4	6	7	9	10	12	15	17	21	22	24	32	33
%Reduction	35	96	25	33	50	9	47	50	39	11	55	27	49	72

Fig. 2. ELISA of roasted peanut and autoclaved roasted peanut at 2.56 atm, 30 min, incubated with sera from patients with actual peanut allergy (Nos. 2, 4, 6, 7, 9, 10, 12, 15, 17) and sera from patients sensitized to peanut (Nos. 21, 22, 24, 32, 33). Percentages of decrease in antigenic activity are shown in the table annexed to Fig. 2.

samples and A_N is the absorbance value of the roasted peanut protein extract (Cabanillas et al., 2010).

The results are summarized in Fig. 2. Roasted peanut IgE reactivity was reduced by autoclaving at 2.56 atm, 30 min treatment in all sera tested. The treatment led to a minimum antigenicity reduction of 9% (in serum from patient 10) and a maximum antigenicity reduction of 96% (in serum from patient 4).

3.3. Ara h 1, Ara h 2 and Ara h 3 following autoclave processing

Solubility of proteins from roasted and autoclaved roasted peanut (1.18 and 2.56 atm, 15 and 30 min) was evaluated by SDS–PAGE extracting proteins into solution by buffer (soluble portion) and the pelleted portion of each sample, brought down by centrifugation following extraction of soluble material with buffer (insoluble portion) (Fig. 3A).

Soluble fractions showed a reduction in the overall level of intact proteins following autoclave treatments compared with insoluble fractions. Specific anti Ara h 1, anti Ara h 2 and anti Ara h 3 antibodies used to identify Ara h 1, Ara h 2 and Ara h 3 molecules in soluble and insoluble fractions of roasted and autoclaved roasted peanut (Fig. 3B) confirmed the results observed in SDS–PAGE. A decrease in the relative Ara h 1, Ara h 2 and Ara h 3 levels could be observed in autoclaved roasted peanut samples with increased pressure and time, in the soluble fraction compared to the insoluble fraction. Nevertheless, these results are more evident in Ara h 1 than Ara h 2 and Ara h 3 immunoblots. The relative level of Ara h 1 increased in the insoluble fraction of autoclaved roasted peanut compared to soluble fraction, and high-molecular-weight-aggregates or oligomers could be observed in the insoluble fraction. However, in spite of these facts, there was a marked decrease in recognition of Ara h 1, Ara h 2 and Ara h 3 in autoclaved roasted peanut at 2.56 atm, 30 min even in the insoluble fractions.

3.4. Susceptibility of autoclaved roasted peanut extract to trypsin

To evaluate digestibility, roasted peanut and autoclaved roasted peanut (1.18 atm, 15 and 30 min) were subjected to trypsin treatment (Fig. 4). SDS–PAGE separation followed by immunodetection with a serum pool from three patients with clinical allergy to peanut (Nos. 7, 12, 15) indicated that autoclaved roasted peanut, compared to roasted peanut, was more extensively digested by trypsin within 10 min of treatment, losing most of its capacity to bind IgE from peanut allergic patients (Fig. 4B).

3.5. CD analysis of the structural alterations

The secondary structure of roasted peanut and autoclaved roasted peanut (1.18 atm and 2.56 atm, 15 and 30 min) was analyzed by CD spectroscopy and the far-UV spectra are shown in Fig. 5. The percentages of α-helix, β-sheet and random coil and/or loops are shown in the table annexed to Fig. 5. The CD spectrum of roasted peanut proteins rendered 50% α-helix, 10% β-sheet, and 40% random coil and/or loops. As can be seen in the case of auto-

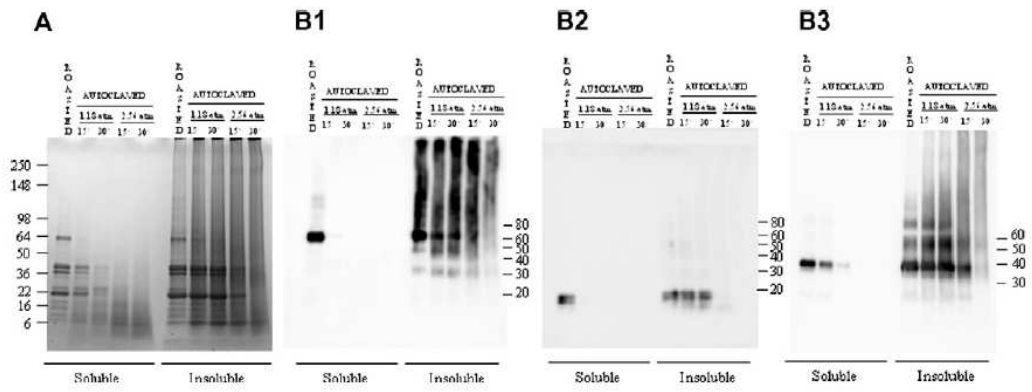


Fig. 3. SDS–PAGE (A) and anti-Ara h 1 (B1), anti-Ara h 2 (B2) and anti-Ara h 3 (B3) western blot of soluble and insoluble fractions of roasted and autoclaved roasted peanut.

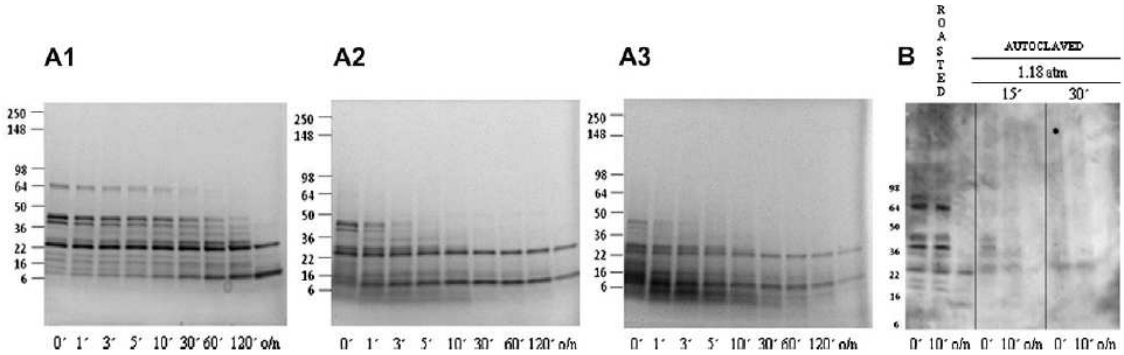
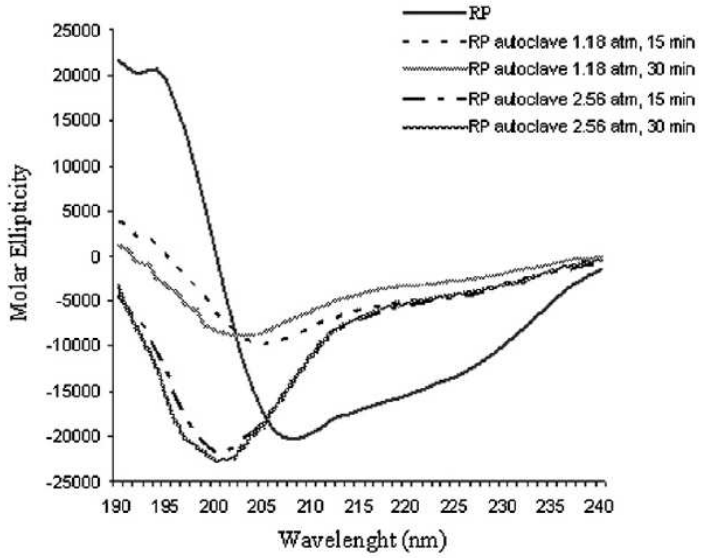


Fig. 4. A. SDS–PAGE analysis of the digestion of roasted peanut (A1), autoclaved roasted peanut 1.18 atm, 15 min (A2) and autoclaved roasted peanut 1.18 atm, 30 min (A3) by trypsin at 0, 1, 3, 5, 10, 30, 60, 120 min and overnight (~15 h). B. Western blot of selected digestion times (0, 10 min and overnight) of roasted peanut and autoclaved roasted peanut using a serum pool from 3 patients with clinical allergy to peanut (Nos. 7, 12 and 15).



	α-helix	β-strand	Random coil and/or loops
RP	50	10	40
RP autoclave 1.18 atm, 15 min	16	24	60
RP autoclave 1.18 atm, 30 min	7	26	67
RP autoclave 2.56 atm, 15 min	15	7	78
RP autoclave 2.56 atm, 30 min	11	9	80

Fig. 5. CD-spectrum of roasted peanut (RP) and autoclaved roasted peanut at 1.18 atm and 2.56 atmospheres (15 and 30 min). Percentages of α-helix, β-strand and random coil and/or loops are shown in the table annexed to Fig. 5.

claved roasted peanut, most of the α-helical structure was lost (11% remaining in autoclaved roasted peanut at 2.56 atm, 30 min), random coil and/or loops increased (80% in autoclaved roasted peanut at 2.56 atm, 30 min) and β-sheets remain.

3.6. Skin prick tests

Table 1 shows the results of skin testing with raw and processed peanut extract samples performed on seven patients with clinical allergy to peanut. All patients had skin reactivity to raw, fried and roasted peanut extract, although six out of seven reactions were positive with roasted peanut. Boiled peanut extract elicited positive reactions in one patient. None of the patients reacted to autoclaved roasted peanut extract. Healthy subjects did not have skin reactivity to any protein extract.

4. Discussion

In this study the effect of different food-processing conditions on peanut allergenicity was investigated. The results showed that IgE immunoreactivity of roasted peanut protein extract decreased significantly at extreme conditions of autoclaving (2.56 atm, 30 min), as shown by *in vitro* experiments of western blot, ELISA and *in vivo* experiments of skin prick tests. These results can be explained by circular dichroism experiments: most of the α-helical structure was lost after autoclave treatments. It is known that many of the IgE binding epitopes in the major allergens of peanuts (i.e. Ara h 1, Ara h 2 and Ara h 3) are located on the α-helical regions of these proteins (Barre, Jacquet, Sordet, Culerrier, & Rougé, 2007; Mueller et al., 2011; Shin et al., 1998). However, although the decrease of IgE-binding proteins is notable, *in vitro* experi-

Table 1
Results of skin prick testing with raw and thermal processed peanuts in patients with clinical allergy to peanut.

SPT (mm) peanut						Immunological and clinical features	
Patient	Raw	Boiled	Fried	Roasted	Autoclaved roasted	CAP-FEIA (kU/L)	Diagnostic challenge
A	4.2	0	3.5	3.5	0	1.26	DBPCFC
B	7.5	0	6.2	5	0	0.2	DBPCFC
C	4.5	0	4.2	2.2	0	0.95	DBPCFC
D	7	3	5.6	5.6	0	6.7	DBPCFC
E	6.5	1.8	5.5	4	0	3.5	DBPCFC
F	7.2	1.5	6	5.5	0	0.8	–[a]
G	6.2	3.7	4.8	5.5	0	4	–[a]

CAP-FEIA, Capsulated hydrolic carrier polymer-fluoro-enzyme immunoassay; DBPCFC, double-blind, placebo-controlled food challenge; SPT, skin prick testing.
[a] Not challenged because of a convincing history of severe anaphylaxis with peanut.

ments showed that 22% of sera recognized some proteins in autoclaved roasted peanut at 2.56 atm, for 30 min, in western blot. ELISA results showed that autoclaved roasted peanut (2.56 atm, 30 min) elicited a decrease of IgE reactivity between 9% and 96%.

These findings suggest that autoclaving produces an important decrease in IgE-binding properties of roasted peanut due to changes in the structure of the proteins. Similar results have been previously reported in other legumes: Malley, Baecher, Mackler, and Perlman (1975) found that autoclaving green pea at 120 °C for 15 min reduced its allergenicity. Also, IgE-binding capacity of lupine allergens was reduced by autoclaving at 2.6 atm, for 30 min, or by DIC (instantaneous controlled pressure drop) at 6 bar (5.9 atm) for 3 min, whereas it was only slightly affected by boiling, microwave, and extrusion-cooking (Alvarez-Alvarez et al., 2005; Guillamón et al., 2008). However, it has been demonstrated that the allergenicity of the almond major protein (amandin) and a peach protein in the nectar (Pru p 1) is maintained after autoclaving at 121 °C, up to 30 min (Brenna et al., 2000; Venkatachalam, Teuber, Roux, & Sathe, 2002).

Autoclaved roasted peanut, compared to roasted peanut, was more extensively digested by trypsin within 10 min of treatment, losing most of its capacity to bind IgE from peanut allergic patients. It has been demonstrated that heat treatments can increase the susceptibility to enzymatic digestion of some allergens (Morisawa et al., 2009). In the present study, circular dichroism experiments showed unfolding of proteins in autoclave treated samples, which makes them more susceptible to digestion. Morisawa et al. (2009) found that B cell epitopes of β-lactoglobulin were impaired by heat treatment, increasing susceptibility to digestion and facilitating the enzymatic cleavage of protein sequences that disrupts linear B cell epitopes.

The degree of processing can dramatically affect the results of digestibility, solubility and other parameters (Maleki, 2004). During processing, proteins can form oligomers, become denatured, degraded, aggregated, cross-linked, fragmented and re-assembled and these changes most often cause a reduction in solubility (Maleki, 2004). In this way, processing can alter the overall IgE binding profiles of a particular extract (Schmitt et al., 2010). It can be hypothesized that the soluble portion of autoclaved roasted peanut samples assessed in this study contains less allergenic proteins or products due to a reduction in the solubility caused by autoclave treatment. For this reason, protein profiles, including peanut allergens: Ara h 1, Ara h 2 and Ara h 3, were assessed in soluble and insoluble fraction of roasted and autoclaved roasted peanut. The results showed that the total level of intact protein decreases with increased pressure and time in both, soluble and insoluble fractions, although soluble fractions showed a higher reduction of proteins than insoluble fractions. Ara h 1, Ara h 2 and Ara h 3 allergens were recognized more extensively by specific antibodies in insoluble fraction than in soluble fraction. Moreover, Ara h 1 was present in higher molecular weight aggregates or oligomers in the insoluble fraction. It has been demonstrated that heating of purified Ara h 1 leads to a more structured secondary conformation of the protein, with an increased content of extended β-sheet structures leading to the formation of large protein complexes or aggregates (Koppelman, Bruijnzeel-Koomen, Hessing, & de Jongh, 1999). However, in the present study, a marked decrease of Ara h 1, Ara h 2 and Ara h 3 allergens was detected at harsh conditions of autoclaving (2.56 atm, 30 min) in both, soluble and insoluble, fractions.

In summary, although further studies are needed to assess the clinical relevance of our findings, we can conclude that autoclaving at 2.56 atm for 30 min produces a significant decrease of IgE-binding capacity of peanut allergens due to changes in their secondary structure. This treatment could be a technique to improve food safety.

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Effect of Instant Controlled Pressure Drop on IgE Antibody Reactivity to Peanut, Lentil, Chickpea and Soybean Proteins

Carmen Cuadrado[a] Beatriz Cabanillas[b] Mercedes M. Pedrosa[a] Mercedes Muzquiz[a] Joseph Haddad[c] Karim Allaf[c] Julia Rodriguez[b] Jesus F. Crespo[b] Carmen Burbano[a]

[a]Departamento de Tecnología de Alimentos, SGIT-INIA, and [b]Servicio de Alergia, Instituto de Investigación Hospital 12 de Octubre, Madrid, Spain; [c]Laboratoire Maîtrise des Technologies Agro-Industrielles, Université de La Rochelle, La Rochelle, France

Key Words
Peanut · Lentil · Chickpea · Soybean · Food allergy · IgE reactivity · Thermal processing · Instant controlled pressure drop · DIC®

Abstract
Background: The use of legume seeds is being expanded in the food industry due to their excellent nutritional and technological properties. However, legumes have been considered causative agents of allergic reactions through ingestion. Previous studies indicated that processing methods combining heat and steam pressure, such as instant controlled pressure drop (DIC®), could decrease allergenicity. The aim of this study was to investigate the impact of DIC treatment on peanut, lentil, chickpea and soybean IgE antibody reactivity. ***Methods:*** Peanut, lentil, chickpea and soybean seeds were subjected to DIC treatment at different pressure and time conditions (3 and 6 bar for 1 and 3 min). Control (raw) and DIC-treated extracts were analyzed by SDS-PAGE and immunoblotting using a serum pool from sensitized patients. ***Results:*** DIC treatment did not affect the total protein content of legume seeds. Nevertheless, modifications of protein profiles after DIC showed a general decrease in IgE binding to legume proteins that was correlated to a higher steam pressure and longer treatment. The immunoreactivity of soybean proteins was almost abolished with treatment at 6 bar for 3 min. ***Conclusions:*** The results demonstrated that DIC treatment produces a reduction in the overall in vitro IgE binding of peanut, lentil and chickpea and a drastic reduction in soybean immunoreactivity.

Introduction

The use of legumes has been expanded in the food industry due to their excellent nutritional and technological properties. In fact, they are recommended as a staple food by health organizations [1], and dieticians are nowadays tending to encourage their consumption in counseling [2]. On the other hand, some typical constraints of legume seed proteins, including the antinutritional and allergenic activities of some of them, limit their wider use as raw food material or ingredients.

Among food allergies, peanut *(Arachis hypogaea)* allergy is one of the most common and severe IgE-mediated reactions to food and is typically lifelong [3]. Therefore, considerable effort has been spent in identifying and characterizing allergens from peanut. The 2 major peanut allergens, Ara h 1 and Ara h 2, are recognized by 70–90% of sensitized subjects [4] and correspond to the

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Correspondence to: Dr. Carmen Burbano
Departamento de Tecnología de Alimentos, SGIT-INIA
Ctra. de La Coruña km 7.5
ES–28040 Madrid (Spain)
Tel. +34 91347 6803, E-Mail burbano@inia.es

vicilin (7S) and 2S families of storage proteins, respectively [5, 6]. Ara h 3 has been considered to play a lesser allergenic role and belongs to the 11S legumin-like proteins [7].

Lentils *(Lens culinaris)* and chickpeas *(Cicer arietinum)* have been reported as causes of IgE-mediated hypersensitivity reactions, particularly in pediatric patients [8–11]. A major lentil allergen, Len c 1, was identified as a 48-kDa vicilin [12, 13]. So far, no chickpea allergen has been identified but several IgE-binding bands (10–70 kDa) have been detected by immunoblotting [10, 11, 14].

To date, 33 soybean *(Glycine max)* proteins have been identified as allergens (from 7 to 71 kDa) [15]. The P34 protein, also referred to as Gly m Bd 30K (thiol protease), has been identified as an immunodominant allergen that shares homology sequence (70%) with Ara h 1. Moreover, the acidic and basic glycinin polypeptides (11S) are allergenic and resistant to processing [16, 17]. The 7S globulin fraction (β-conglycinin) includes the α and β subunits, which are also allergenic proteins [18].

The effects of thermal processing on food protein allergenicity have been reviewed in several recent articles [19–21]. The molecular basis of changes in allergenic activity is the inactivation or destruction of epitope structures, the formation of new epitopes or enhanced access to cryptic epitopes by denaturation of the native allergen, which may increase or decrease the IgE immunoreactivity of a protein [22].

Previous studies have reported that boiling in an autoclave (2.6 bar for up to 30 min) markedly reduced lupin, lentil and chickpea allergenicity [23, 24]. Moreover, the use of instant controlled pressure drop (DIC®) treatment (6 bar for 3 min), combining heat and steam pressure as in autoclaving, greatly decreased IgE reactivity of the heat-resistant major lupin allergens (Lup-1 and Lup-2) [25, 26]. Taking into account these findings, the aim of this study was to investigate the impact of DIC treatment of raw and roasted peanuts, lentils, chickpeas and soybeans on IgE reactivity using a serum pool from sensitized patients.

Materials and Methods

Plant Material

Raw and roasted peanuts (*A. hypogaea* var. Virginia) were purchased from Aperitivos Medina (Madrid, Spain); lentil seeds (*L. culinaris* var. Magda) were provided by the Instituto Técnico Agronómico Provincial (Albacete, Spain), and chickpea seeds (*C. arietinum* var. Athenas) and soybean seeds (*G. max* var. Ostrumi) were obtained from the Instituto de Formación e Investigación Agraria y Pesquera (Córdoba, Spain). All seeds were subjected to DIC treatment.

DIC Treatment

DIC treatment was carried out following a factorial experimental design previously described [27, 28]. Briefly, the moistened product is placed in a processing chamber and exposed to steam pressure (up to 8 bar) at high temperature (up to 170°C) over a relatively short period of time (a few seconds to some minutes). This high-temperature/short-time stage is followed by an instant pressure drop towards a vacuum at about 50 mbar. This abrupt pressure drop, at a rate of $\Delta P/\Delta t$ higher than 5 bar/s, simultaneously provokes autovaporization of part of the water in the product and an instantaneous cooling of the products, which stops thermal degradation. Whole seeds of raw and roasted peanuts, lentils, chickpeas and soybeans were treated at different pressures for different periods of time and with different initial water content conditions. For raw and roasted peanuts, a central composite design of 12 points was used with 4 repetitions of the central point, while for the other legumes, a 22-point central composite design with 10 repetitions of the central point was used. From the parameters used for all these DIC-treated samples, treatment at 3 and 6 bar for 1 and 3 min with constant initial water content of 50 g of water per 100 g of dry matter was selected for SDS-PAGE and immunoblotting analysis.

Protein Extracts

Control (unprocessed) and DIC-treated seeds were milled to pass through a 1-mm sieve (Cyclotec 1093, Tecator, Sweden), and the flour was defatted with n-hexane (34 ml/g of flour) for 4 h, shaken and air dried after filtration. Defatted flour of raw and roasted peanut samples was extracted twice in a solution of 0.05 M Tris-HCl buffer (pH 8.0) with salt (0.5 M NaCl). The defatted flours of lentil, chickpea and soybean samples were extracted (×2) with 0.1 M PBS buffer (pH 7.4) plus 0.15 M NaCl. All the extractions were carried out at 1:10 (w/v) for 1 h at 4°C with stirring. The extracts were clarified by centrifugation at 27,000 *g* for 30 min at 4°C, and the supernatants were dialyzed against distilled H_2O for 24 h at 4°C using a dialysis membrane (Spectra/Por, Serva, Heidelberg, Germany) with a cut-off of 3.5 kDa and then freeze dried. The soluble protein content in each extract was measured by the Bradford dye binding assay (Bio-Rad, Hercules, Calif., USA) using bovine serum albumin (Sigma, St. Louis, Mo., USA) as standard. The total nitrogen was determined using the Kjeldahl procedure [29], and the total crude protein was calculated as N × 5.45 [30].

Patient Sera

A serum pool with specific IgE to peanut (74.0 kU/l), lentil (31.3 kU/l), chickpea (29.0 kU/l) and soybean (13.9 kU/l), quantified by the CAP-FEIA system (Pharmacia Diagnostic, Uppsala, Sweden), was employed. There is no further clinical information available for this serum pool. Serum from a patient with specific IgE to *Anisakis* spp. (9.09 kU/l) and a total serum IgE value of 53.4 kU/l was used as a negative control.

Protein Electrophoresis and IgE Immunoblot Experiments

SDS-PAGE was performed according to Laemmli [31]. Samples (20 μg of protein per lane) were mixed with Laemmli sample buffer (Bio-Rad) and 2-mercaptoethanol (Bio-Rad), heated at

90°C for 10 min and electrophoresed in 4–20% Tris-HCl linear gradient precast gel (Bio-Rad). Proteins were visualized with Coomassie Brilliant Blue R250 staining. Western blot was performed by electrophoretic transfer to a nitrocellulose membrane (Whatman, Dassel, Germany). After blocking with 3% (w/v) non-fat milk and 0.1% (v/v) Tween 20 in PBS (pH 7.4; blocking buffer), the membranes were incubated overnight with the serum pool or negative control serum (1:100 dilution), washed and then treated with mouse anti-human IgE monoclonal antibody HE-2 ascitic fluid (1:5,000 dilution for 1 h; ALK-Abello, Madrid, Spain). After washing, a goat anti-mouse IgG peroxidase-conjugated antibody (1:2,500 dilution for 1 h; Pierce, Rockford, Ill., USA) was added. Detection of IgE-binding components was achieved by means of enhanced chemiluminescence, according to the manufacturer's instructions (Amersham, UK).

Results

Protein Content

The total crude protein content was analyzed in the defatted legume flour, and the mean values were compared by Duncan's test with a significance level of $p < 0.05$. The results show that the crude protein content of untreated raw peanuts (42.96 g/100 g dry matter) was significantly lower than that in the DIC-processed samples, which ranged from 43.81 g/100 g at 3 bar for 1 min to 45.95 g/100 g at 3 bar for 3 min. Untreated roasted peanuts had 45.43 g/100 g, which was not significantly different from the samples treated with DIC at 3 bar (1 and 3 min) and 6 bar for 1 min (from 46.70 to 46.99 g/100 g), but was significantly lower than the sample treated with DIC at 6 bar for 3 min (49.43 g/100 g). There were no significant differences between the total protein content of lentil (22.85 g/100 g), chickpea (19.7 g/100 g) and soybean control samples (35.91 g/100 g) and the corresponding DIC-treated ones.

Effect of DIC on Peanut Allergenic Proteins

The SDS-PAGE patterns of the protein extracts of raw and roasted peanuts before (control) and after DIC treatments are shown in figure 1. The extracts were also analyzed by IgE immunoblot using the serum pool. SDS-PAGE and IgE immunoblot band patterns of untreated raw and roasted peanuts were similar (fig. 1), showing multiple immunoreactive proteins, such as a protein around 65 kDa (putative Ara h 1), several bands ranging from 25 to 37 kDa, which very likely correspond to Ara h 3 fragments, and 1 band with a molecular weight around 17 kDa, presumably corresponding to Ara h 2 [4]. In addition, allergenic proteins of lower molecular weight (<16 kDa) were present.

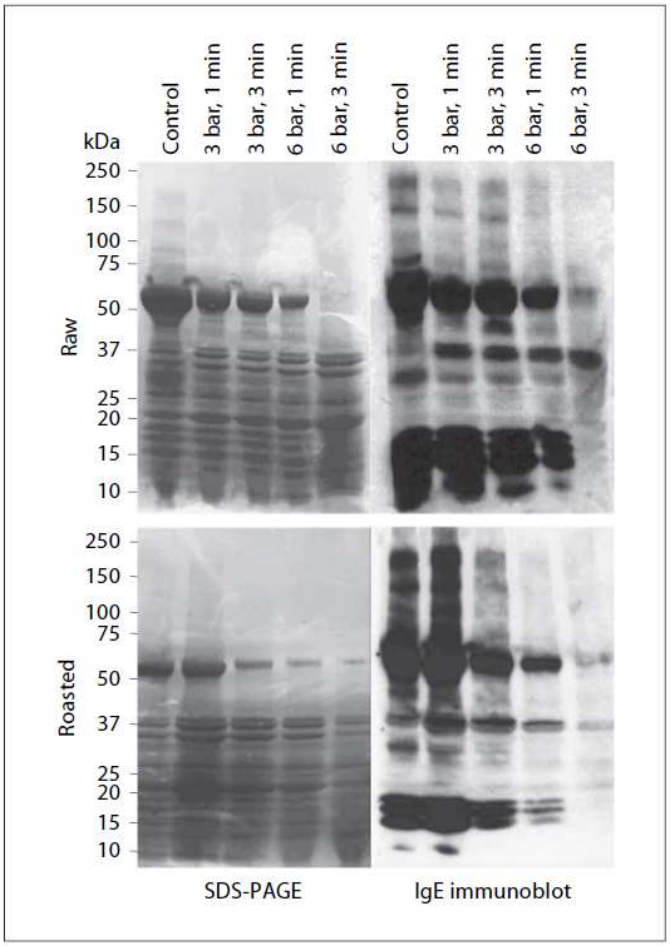

Fig. 1. SDS-PAGE patterns and IgE immunoblots of control and DIC-processed samples of raw and roasted peanuts (2 replicates were performed). The serum pool used was from sensitized subjects (specific IgE to peanut: 74 kU/l; 20 μg of protein per lane).

Following DIC treatment at 3 bar for 1 and 3 min, no changes in the SDS-PAGE protein band pattern and minimal changes (decrease/increase) in the immunoreactive band pattern of raw and roasted peanuts could be seen (fig. 1). At 6 bar for 1 and 3 min (fig. 1), a decrease in the band intensity in the range 15–65 kDa was observed; this change was more marked in roasted than in raw peanuts. More precisely, the 65- and 37-kDa protein bands (putative Ara h 1 and Ara h 3, respectively) were less intense in roasted than in raw peanuts, although they were still visible (fig. 5a, b).

Effect of DIC on Lentil Allergenic Proteins

Figure 2 shows SDS-PAGE and Western blot patterns of control and DIC-processed lentil extracts. Control len-

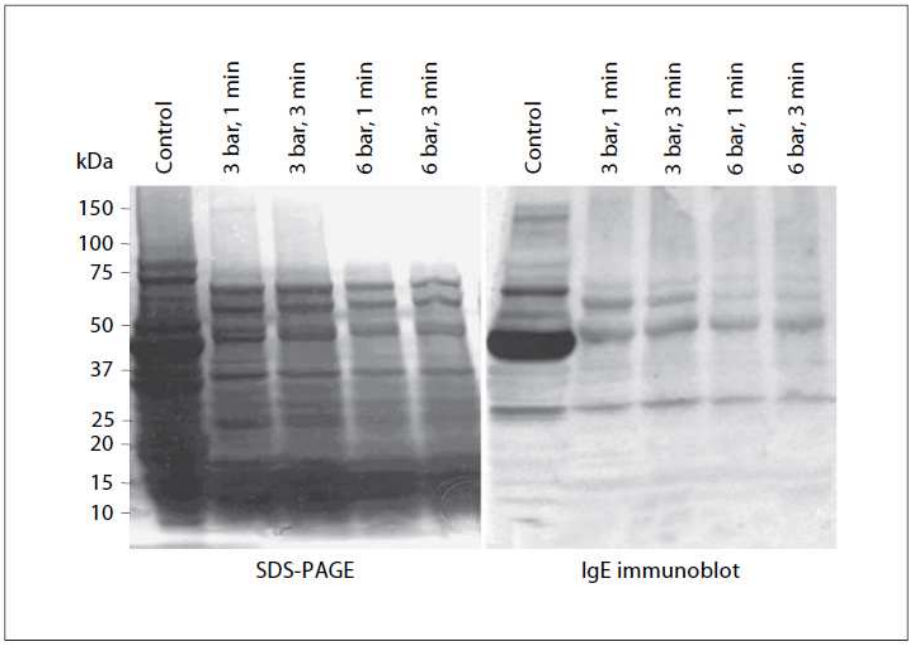

Fig. 2. SDS-PAGE patterns and IgE immunoblots of control and DIC-processed samples of lentils (2 replicates were performed). The serum pool used was from sensitized patients (specific IgE to lentil: 31.3 kU/l; 20 μg of protein per lane).

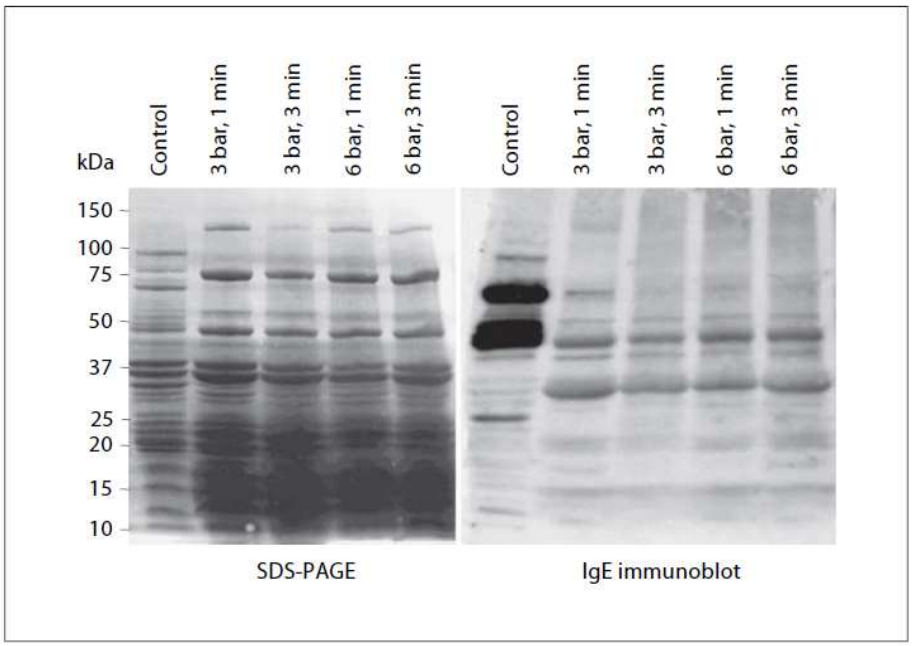

Fig. 3. SDS-PAGE patterns and IgE immunoblots of control and DIC-processed samples of chickpeas (2 replicates were performed). The serum pool used was from sensitized subjects (specific IgE to chickpea: 29 kU/l; 20 μg of protein per lane).

til was composed of numerous immunoreactive bands with molecular weights between 10 and 140 kDa, including a protein of 48 kDa (putative major allergen Len c 1). DIC processing at 3 and 6 bar for 1 and 3 min produced a marked decrease in overall immunoreactivity. Only 4 heat-stable bands were still present at harsh processing conditions of steam pressure and time (fig. 5c).

Effect of DIC on Chickpea Allergenic Proteins

SDS-PAGE patterns of protein extracts from untreated (control) and DIC-treated chickpeas are shown in figure 3, which also shows IgE immunoblotting analysis of these extracts using a serum pool from subjects with specific IgE to chickpea. Untreated chickpeas showed numerous IgE-binding proteins with molecular weights between 12 and 82 kDa. The immunoreactive band pattern

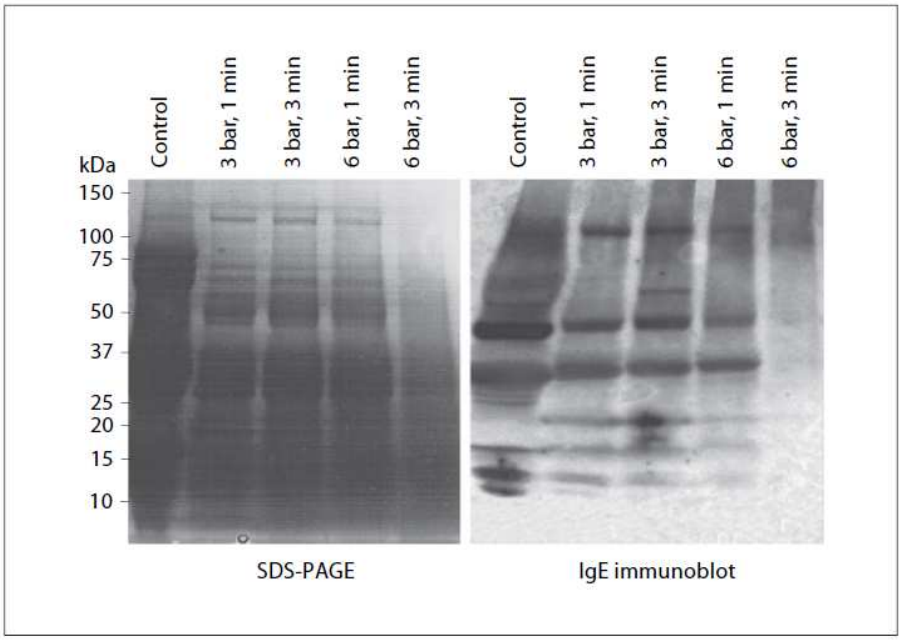

Fig. 4. SDS-PAGE patterns and IgE immunoblots of control and DIC-processed samples of soybeans (2 replicates were performed). The serum pool used was from sensitized patients (specific IgE to soybean: 13.9 kU/l; 20 μg of protein per lane).

after DIC treatments showed a marked decrease in the number and intensity of IgE-binding proteins; however, there were no apparent distinctions between the different DIC conditions applied, and only a few heat-stable immunoreactive proteins were still present (fig. 5d).

Effect of DIC on Soybean Allergenic Proteins

SDS-PAGE and Western blot using a serum pool with specific IgE to soybean were performed with control and DIC-processed soybean samples (fig. 4). In untreated soybeans, the major allergens and other minor immunoreactive proteins between 13 and 119 kDa were detected. The DIC process applied at 3 bar for 1 and 3 min and at 6 bar for 1 min produced a similar reduction of IgE-immunoreactive bands (fig. 4). However, after DIC at 6 bar for 3 min, almost all immunoreactive proteins were eliminated (fig. 5e).

Discussion

DIC processing can retexture biological products, improving their functional properties for use in industrial processes [32], but usually does not affect the total crude protein content. In the present study, as expected [25, 32], there was no reduction in the total protein content in DIC-treated legume seeds compared with the control samples. However, the soluble protein content values measured in each legume extract (data not shown) indicated that more protein remained in the residue as unextractable material after DIC processing than in the control legume samples, a fact which is in agreement with previous experiments with lupins [25] and with reports indicating that heating promotes protein denaturation and aggregation and therefore can make some seed proteins unextractable and causes them to remain in the residue [33].

Significant alterations in protein structure may occur during heat treatment, the nature and extent of which depend on the temperature and duration of the thermal processing. Heat treatment can destroy epitopes by denaturation of proteins, with the result that only linear epitopes are available for binding to antibodies [33]. Most investigations have focused on the influence of thermal processing on peanut [6, 33–35]. Maleki and Hurlburt [36] showed that roasting, in addition to the effect of the Maillard reaction products, induces structural modifications reducing the solubility of the peanut allergens Ara h 1 and Ara h 2 and inducing their resistance to digestion, a fact which could contribute to the enhanced IgE-binding capacity of roasted peanut.

At present, little is known about the effect of thermal treatment under pressure, such as autoclaving and DIC, on allergenic proteins. According to our previous studies, lupin allergenicity was abolished by autoclaving at 2.6 bar for 30 min [23] or by DIC treatment at 6 bar for 3 min [25],

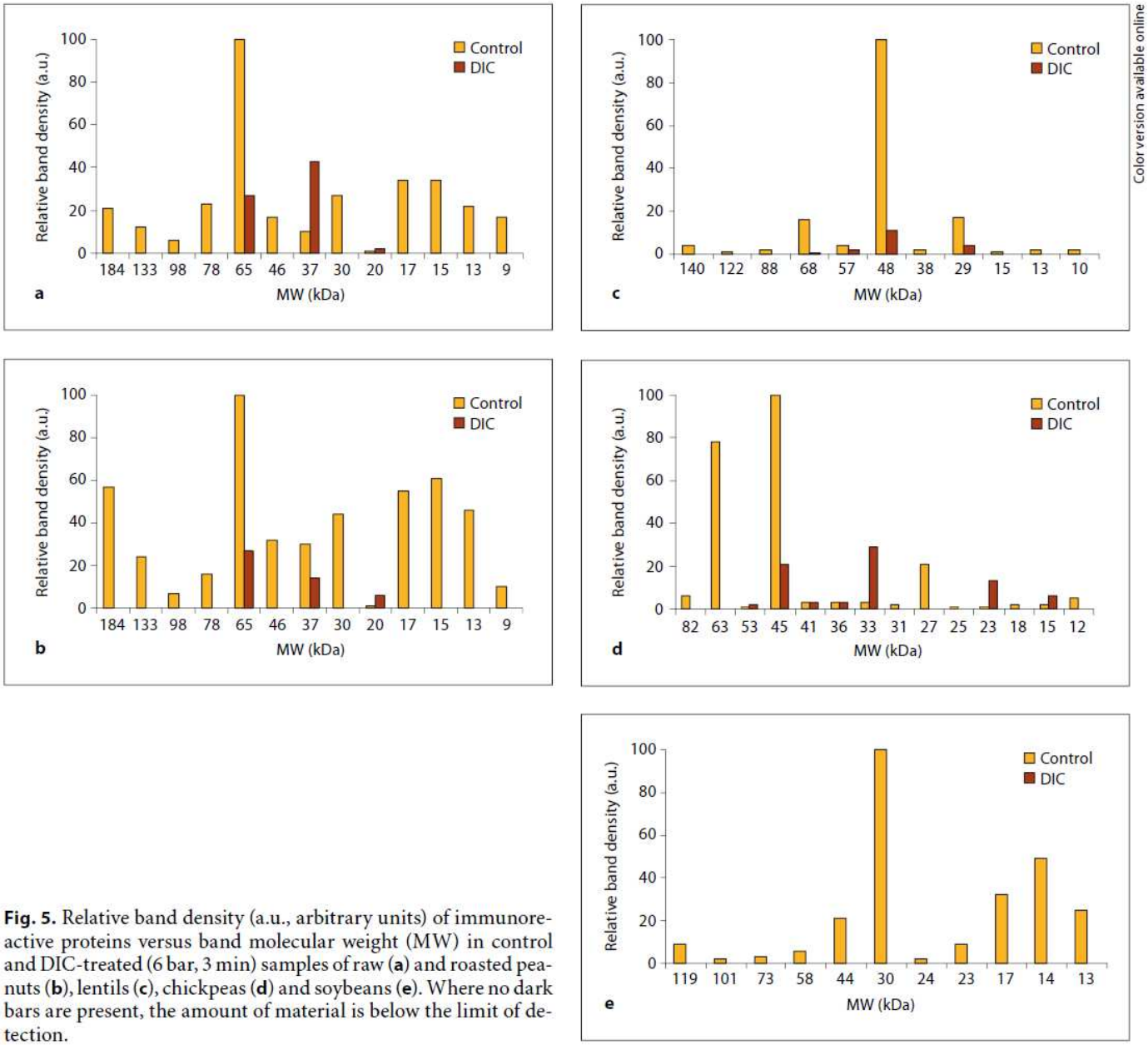

Fig. 5. Relative band density (a.u., arbitrary units) of immunoreactive proteins versus band molecular weight (MW) in control and DIC-treated (6 bar, 3 min) samples of raw (**a**) and roasted peanuts (**b**), lentils (**c**), chickpeas (**d**) and soybeans (**e**). Where no dark bars are present, the amount of material is below the limit of detection.

whereas it was only slightly affected by boiling and microwave and extrusion cooking [23]. These results support the high thermal resistance of the lupin allergens Lup-1 (34.5 kDa) and Lup-2 (20 kDa) [26].

The present results indicated that DIC treatment at 3 bar for 1 and 3 min and 6 bar for 1 min produced little modification of SDS-PAGE patterns or IgE-binding capacity of raw and roasted peanut proteins. DIC treatment at harsh conditions (6 bar for 3 min) produced in both samples a remarkable decrease in the protein bands of 65 kDa (Ara h 1), and no immunoreactive bands lower than 20 kDa were recognized. However, some bands (e.g. 37 kDa, Ara h 3) behave differently in raw and roasted peanuts (fig. 5a, b). Taking into account the reduction of in vitro immunoreactivity obtained at the highest pressure and time applied, the DIC technique seems to have more effect on the immunoreactivity of roasted peanut proteins, which seem to be even less immunoreactive than DIC-treated raw peanuts.

Some data also exist on adverse reactions to temperate legumes, including lentils and chickpeas. Boiling lentils did not reduce immunoreactivity [12, 37]. Chickpea allergy has been reported in Spain, where this legume is commonly consumed [10], and recently a high degree of cross-reactivity among lentils, chickpeas and peas has been observed [38].

Regarding the effect of thermal treatment on lentil and chickpea seeds, our previous studies show that autoclaving at 2.6 bar for 30 min produced a significant decrease in the IgE-binding capacity of lentil and chickpea allergens, although extremely resistant immunoreactive proteins still remained in both legumes even after this heat and pressure process [24]. In this study, extreme DIC treatments produced a similar pattern of IgE-binding proteins to that observed with autoclave at harsh conditions and, therefore, a parallel reduction in the overall IgE immunoreactivity. However, similarly, some heat-stable allergenic proteins were still detected in the Western blot analysis. These results confirmed the thermostability of some lentil and chickpea allergens.

Due to the commercial use of soybean protein-based food products, which frequently involves thermally processed proteins, it is important to investigate the effect of heat treatment on the main allergens of this legume. Burks et al. [39] did not find a significant decrease in IgE binding after heating soy proteins at various temperatures and time intervals. Wilson et al. [15] concluded that several procedures are needed to eliminate soy allergenicity, particularly of P34, the major allergenic protein. Our present results showed that although DIC treatment at 3 bar for 1 and 3 min resulted in a slight reduction of the soybean immunoreactive bands, when pressure was increased up to 6 bar and applied for 3 min, the overall in vitro IgE reactivity was markedly decreased, similarly to the effect of autoclaving (2.6 bar, 30 min) on this legume [40]. The DIC technique employed has a strong effect on the immunoreactive proteins of soybean with a short processing time (3 min), a fact which might represent an advantage for future potential applications in the food industry. Elimination of allergenic proteins via processing could eventually enhance the safe use of soybean products, making them available to soy-sensitized individuals. According to Thomas et al. [41], food processing may impact the allergenicity potential of proteins; however, there are no general rules regarding how different allergenic foods respond to physical, chemical or biochemical processing methods. In some, the epitopes are destroyed, but in others they are unaltered. The modifications eventually result in loss of organized structure and protein denaturation. However, it has been demonstrated that the degree of processing can dramatically affect the results of digestibility, solubility and other parameters [42]. During processing, proteins can form oligomers, become denatured, degraded, aggregated, cross-linked, fragmented and reassembled, and these changes most often cause a reduction in solubility [42]. In this way, processing can alter the overall IgE-binding profiles of a particular extract [33]. Therefore, further studies would be needed to evaluate the effects of these treatments on the insoluble protein fraction of these legumes.

This study supports our previous findings regarding the effect of DIC treatment (steam pressure and high temperature) on heat-stable lupin allergens. The present results show that DIC treatment is able to produce an important decrease in the overall immunoreactivity of peanut, lentil and chickpea extracts and a marked reduction in IgE recognition of soybean protein extract. These DIC-treated protein extracts could constitute an alternative to intact proteins in the development of different food products.

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VI

Letters

SYSTEMIC IgE-MEDIATED REACTION TO A DIETARY SLIMMING BAR

Leaves from the prickly pear cactus *Opuntia ficus-indica* are consumed as staple food in some countries. Heat-desiccated components derived from these cactus leaves are included in some dietary products because of their alleged beneficial effects on blood lipids.[1,2]

A 33-year-old woman reported an acute episode of oral, pharyngeal, and cutaneous pruritus, facial swelling, conjunctivitis, dysphonia, difficulty swallowing, dyspnea, wheezes, general malaise, and hypotension a few minutes after ingestion of half (22.5 g) a slimming bar. She was treated in the emergency department with epinephrine, intravenous saline, and corticosteroids. The labeled components of the dietetic bar were as follows: "milk and wheat proteins, sugar, soy lecithin, vitamins A, B_1, B_2, B_6, C, D_3, folate, D_3 and NeOpuntia." The patient has tolerated further ingestions of milk, wheat, soy, and vitamins.

After providing an informed consent form, the patient underwent testing by the skin prick test (SPT) method with the dietetic bar, which elicited a positive response: 13 × 5-mm wheal (30 × 20-mm erythema). A SPT with lyophilized NeOpuntia provided by the manufacturer (Bio Serae Laboratories, Bram, France) was performed with serial dilutions (×10), starting at 1 mg/mL, which elicited a negative result. Concentrations of 10 mg/mL and 100 mg/mL induced a 3 × 5-mm wheal (9 × 8-mm erythema) and a 5 × 4-mm wheal (6 × 10-mm erythema), respectively. The results of a SPT with the dietetic bar and NeOpuntia were negative in 4 healthy control subjects. NeOpuntia is described by the manufacturer as a patented "gentle, solvent-free, chemical-free process including drying through heat treatment" product from fresh *O ficus-indica* leaves; therefore, SPTs with raw and heat-dried (90°C and 150°C) *O ficus-indica* fresh leaves were performed, with negative results in all cases. Because the patient had not consumed prickly pears, a SPT was performed, which produced negative results, and an open oral challenge with 2 pieces of fresh prickly pear fruit (110 g) was performed, without any adverse reaction. Extracts from *O ficus-indica* leaves and NeOpuntia were obtained, and enzyme-linked immunoassays were performed in duplicate to determine specific IgE in the patient's serum (1:2 dilution) to both extracts (30 μg/mL). Blocking buffer (0.056 OD units) and serum samples from 2 healthy subjects (0.177 OD units to NeOpuntia and 0.286 OD units to *O ficus-indica* for subject 1 and 0.111 OD units to NeOpuntia and 0.232 OD units to *O ficus-indica* for subject 2) were used as negative controls. The highest value was considered as a cutoff point for positivity. In this experiment, specific IgE levels higher than 0.286 OD units (OD to *O ficus-indica* using sera from healthy subject 1) were considered positive. The results in the patient's serum test showed positive IgE levels to NeOpuntia (0.541 OD units) and negative IgE levels to *O ficus-indica* (0.27 OD units).

Information about allergic reactions to *O ficus-indica* or its processed products is scarce. Yoon et al[3] reported a case of allergic contact dermatitis with prickly pear fruit in a 43-year-old woman who had applied a sliced fruit of *O ficus-indica* to her skin as a folk remedy. Patch testing performed with the fruit showed a strong positive reaction in the patient. A recent study described a patient reporting facial and labial angioedema, erythema, a disseminated micropapular eruption in the trunk and wrists, nausea, and dyspnea 5 minutes after eating 2 cactus fruits. The patient was treated in the emergency department. Prick-prick tests performed with cactus fruit pulp and skin elicited positive results. In the immunoblot, several immunoreactive bands were detected: one with a molecular weight of 15 kDa and most between 37 and 100 kDa.[4] To our knowledge, allergic reactions elicited by ingestion of *O ficus-indica* leaves or NeOpuntia have not been reported so far. Our investigation suggests NeOpuntia as the offender in a patient with an IgE-mediated systemic reaction to a slimming bar containing this product. Skin testing and enzyme-linked immunosorbent assay showed positive results with NeOpuntia; however, when these tests were performed with raw and heat-dried, fresh *O ficus-indica* leaves, the results were negative. It might be hypothesized that the patented thermal processing to obtain NeOpuntia could be different from the heat treatment used in this study and might have the potential to elicit an allergic response in this patient.

Beatriz Cabanillas Martín, MS*
Jesús F. Crespo, MD, PhD*
Carmen Burbano, PhD†
Julia Rodríguez, MD, PhD*

*Servicio de Alergia
Hospital Universitario 12 de Octubre
†Departamento de Tecnología de Alimentos
SGIT-INIA
Madrid, Spain
bcabanillas.hdoc@salud.madrid.org

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HISTAMINE AND METHACHOLINE CHALLENGE CUT POINTS

Direct bronchoprovocation challenges with histamine or methacholine are, with some caveats, highly sensitive tests for current symptomatic asthma. The original tidal breathing method defined a cut point for the provocation concentration causing a 20% decrease in forced expiratory volume in 1 second (FEV_1) (PC_{20}) as 8 mg/mL.[1] Because the PC_{20} measurement is imprecise, with best-case repeatability of ±1 doubling concentration, the American Thoracic Society defined 8 mg/mL ± 1 concentration (IE, 4-16 mg/mL) as a *borderline* range, with a cut point of 16 mg/mL defined as the level above which a histamine or methacholine challenge result would be defined as negative.[2]

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VII

CLINICALLY RELEVANT CROSS-REACTIVITY BETWEEN LATEX AND PASSION FRUIT

A 36-year-old woman reported that she had experienced generalized urticaria, oropharyngeal pruritus, tongue swelling, dysphagia, dysphonia, cough, rhinorrhea, sneezing, lacrimation, and ocular itching immediately after drinking a 330-mL can of mango and passion fruit juice. A year before the reaction, she had experienced an episode of oropharyngeal pruritus minutes after eating a chestnut. She reported local pruritus and erythema whenever she used latex gloves during the last 5 years. The patient eats avocado and banana with no adverse symptoms.

After obtaining informed consent, we conducted skin prick testing with passion fruit–mango juice, passion fruit, mango, and chestnut by the prick-prick method. Then, 9- and 22-mm wheals were observed in the patient after testing with passion fruit–mango juice and passion fruit, respectively; skin test results were negative with mango and chestnut. The skin prick test result was positive with a latex extract (wheal diameter, 26 mm). Specific IgE levels (UniCAP assay; Phadia, Uppsala, Sweden) to passion fruit, chestnut, latex, and rHev b 6.02 (hevein) were 2.17, 0.72, 10.50, and 14.30 kUA/L, respectively. No specific IgE was detected with mango extract or rHev b 1, 3, 5, 8, 9, and 11. By UniCAP-inhibition experiments, the patient's IgE antibody to passion fruit was completely inhibited by latex extract (0.1 mg/mL), but IgE binding to latex and rHev b 6.02, inhibited with passion fruit (1 mg/mL), yielded a partial inhibition (58% and 63%, respectively). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis/immunoblotting studies of passion fruit extract showed IgE binding components of 16 to 54 kDa. A complete dose-dependent inhibition of passion fruit was observed after preincubation with latex extract (Fig 1).

A double-blind, placebo-controlled food challenge was performed with chestnut. Increasing doses of a liquid vehicle (custard, milk, coffee, sugar, and wheat germ) containing (verum) or not (placebo) ground chestnut were administered on separate days. A cumulative dose of 5.4 g of chestnut elicited a clinical response. The result of an open food provocation with fresh mango was negative. Symptoms induced by the offending juice precluded further challenges with passion fruit.

Cross-reacting IgE antibodies recognizing latex and passion fruit allergens were first demonstrated by radioallergosorbent inhibition tests.[1] In addition, putative class 1 chitinases have been reported to be relevant cross-reactive components in foods associated with the latex-fruit syndrome.[2] Our investigation confirms the cross-reactivity between passion fruit and latex in a patient with IgE (to hevein) from latex and its clinical relevance. Therefore, it could be important to evaluate the risk of reaction to passion fruit in latex-allergic patients because exposure to this exotic fruit, even as a component of soft drinks, could not be exceptional and might be a risk for allergic reactions.

Beatriz Cabanillas, PhD
Julia Rodríguez, MD, PhD
Natalia Blanca, MD, PhD
M. Aranzazu Jiménez, MD
Jesús F. Crespo, MD, PhD

Servicio de Alergia
Hospital Universitario 12 de Octubre
Madrid, Spain
bcabanillas.hdoc@salud.madrid.org

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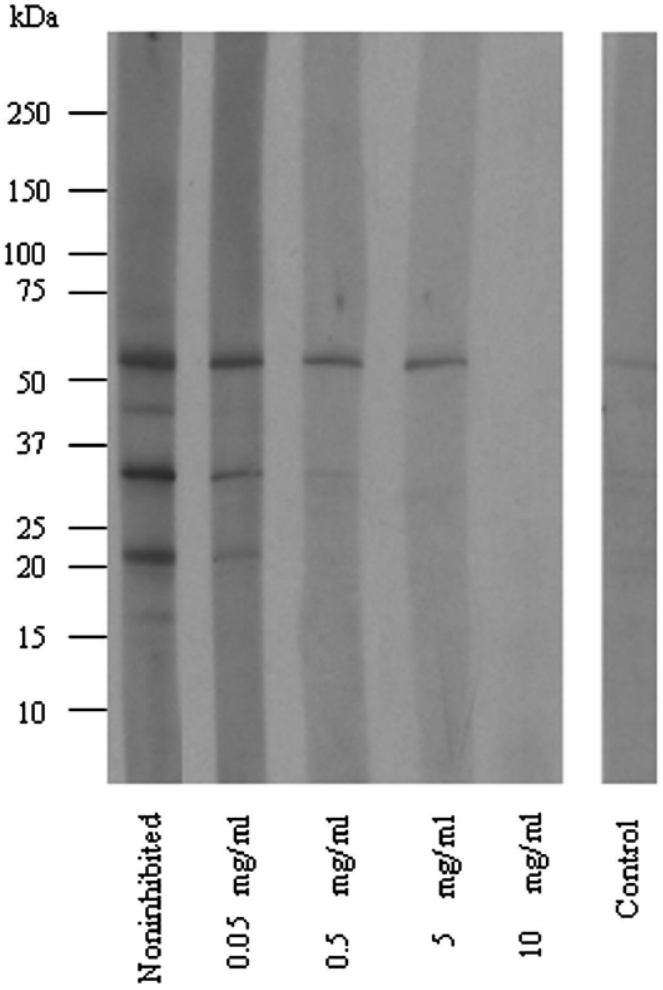

Figure 1. Inhibition of passion fruit immunoblot by latex extract at different concentrations. The control indicates inhibition of passion fruit immunoblot by passion fruit extract (1 mg/mL).

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FATAL ANAPHYLACTIC SHOCK AND *TAENIA SOLIUM* INFESTATION: A POSSIBLE LINK?

While performing a retrospective analysis of forensic-autopsied cases in the last 25 years, we found an interesting case of anaphylaxis that occurred in 1985. A 26-year-old, healthy, male farmer was found dead. Death occurred during sleep and did not seem linked to any previous known diseases. The man's relatives and physician reported that the man had not previously presented with any type of allergy or history of drug or alcohol abuse.

Cadaveric examination showed copious dark blood with clots in the heart cavities; dark red surface of the lungs with a moderate

Disclosures: Authors have nothing to disclose.

VIII

Letters

UNCOVERED REACTIVITY TO LUPINE IN LENTIL-ALLERGIC PATIENTS

Lupine (*Lupinus albus,* Leguminoseae) is well documented as causing food allergy.[1] Cross-reactivity among lupine, peanut, and other Leguminoseae has been reported.[1–5] Cross-reactive allergens to almond have been described in lupine-allergic patients' sera.[6] Herein, we describe the immunologic and clinical findings of lupine reactivity in 6 lentil-allergic adults.

A 20-year-old woman (patient 1) allergic to lentil and peanut reported itchy mouth, difficulty swallowing, tongue swelling, disseminated urticaria, asthma, and abdominal pain on lupine seed ingestion. Skin prick-prick testing (SPPT) with lupine seeds elicited a positive result (9.5-mm wheal). Specific IgE to lupine (CAP-FEIA) was 7.61 kUA/L. The severity of the reaction precluded further oral challenges. These clinical findings prompted us to evaluate a possible cross-reactivity between lupine and lentil.

Five consecutive lentil-allergic adults (4 women; median age, 48 years; age range, 18-54 years; double-blind, placebo-controlled food challenge (DBPCFC)–positive or a convincing severe reaction) were recruited during a 5-year period. They had not ingested lupine previously. Results of SPPT to lentil were positive in all patients. Median specific IgE to lentil was 6.13 kUA/L (range, 0-30.7 kUA/L). All the patients signed an informed consent form for evaluation of lupine allergy. Patient 2, a 49-year-old man, and patient 3, a 27-year-old woman, both nonallergic to peanut, showed positive SPPT and specific IgE to lupine (11- and 7.5-mm wheals and >100 and 1.42 kUA/L, respectively). Both patients underwent DBPCFC with lupine flour.[7] Patient 2 showed intense oropharyngeal pruritus, difficulty swallowing, and dyspnea with a 0.6-g cumulative dose. Patient 3 showed oropharyngeal pruritus, nausea, and intense abdominal pain with a 10-g cumulative dose.

Patient 4 showed a positive SPPT reaction (4-mm wheal) and specific IgE (2.87 kUA/L) to lupine. Results of SPPT and specific IgE to lupine were negative in patients 5 and 6. Open oral challenges with lupine seeds (25-g cumulative dose) elicited a negative result in these 3 patients. Results of lentil and lupine reactivity are summarized in Table 1.

Lentil and lupine extracts were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in 4% to 20% Tris hydrochloride linear gradient precast gel. IgE immunoblot with sera from the 3 lentil- and lupine-allergic patients (patients 1, 2, and 3) (1:10 dilution) were performed as described elsewhere.[8] All sera reacted to 49-, 36-, 18-, and 16-kDa proteins in lentil immunoblot. IgE immunoblot to lupine showed IgE binding to a 72- to 73-kDa protein in all sera. Two of 3 sera recognized a 36-kDa protein. To evaluate cross-reactivity between lentil and lupine, immunoblot inhibitions were performed with sera from patients 2 and 3, both DBPCFC positive to lupine. Serum from patient 2 showed dose-dependent, almost complete inhibition by lupine of all bands in the lentil immunoblot. No inhibition of the lupine immunoblots by lentil was obtained. Serum from patient 3 showed complete inhibition of lupine by lentil in all concentrations used. No inhibition of reactive IgE bands was observed in lentil immunoblot with lupine as inhibitor. Results are shown in Figure 1.

These findings show lupine sensitization in 4 of 6 lentil-allergic adults and clinical reactivity to lupine in 3 of 6. To our knowledge, only an isolated case of lupine-induced anaphylaxis in a patient reporting symptoms with lentil had been described.[3] Immunoblot inhibition in the 2 DBPCFC-positive patients showed opposite re-

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Table 1. Immunologic and Clinical Reactivity to Lentil and Lupine in 6 Lentil-Allergic Patients

Patient No.	Lentil					Lupine				
	SPPT, mm	CAP-FEIA, kU/L	Symptoms	Diagnostic challenge	Allergy	SPPT, mm	CAP-FEIA, kU/L	Symptoms	Diagnostic challenge	Allergy
1	8.5	20.2	U, AE, P, E, OAS, T, DS, A, G	[a]	Yes	9.5	7.61	U, AE, P, E, OAS, DS, T, GI, A, G	[a]	Yes
2	5.0	6.1	P, DY, A	[a]	Yes	11.0	>100	Not consumed	DBPCFC	Yes
3	16.5	30.7	U, P, OAS, R, O, A	[a]	Yes	7.5	1.42	Not consumed	DBPCFC	Yes
4	12.0	7.8	OAS, DS, T, GI, A, G	[a]	Yes	4.0	2.87	Not consumed	OFC	No
5	5.0	0	U, AE, P, E, A	DBPCFC	Yes	0	0	Not consumed	OFC	No
6	9.0	0	U, AE, P, E, GI, A	[a]	Yes	0	0	Not consumed	OFC	No

Abbreviations: AE, angioedema; A, asthma; DBPCFC, double-blind, placebo-controlled food challenge; DS, difficulty swallowing; DY, dysphonia; E, erythema; G, general malaise; GI, gastrointestinal; OAS, oral allergy syndrome; O, ocular; OFC, open food challenge; P, pruritus; R, rhinitis; SPPT, skin prick-prick testing; T, tongue swelling; U, urticaria.

[a] Not challenged because of a convincing history of severe anaphylaxis.

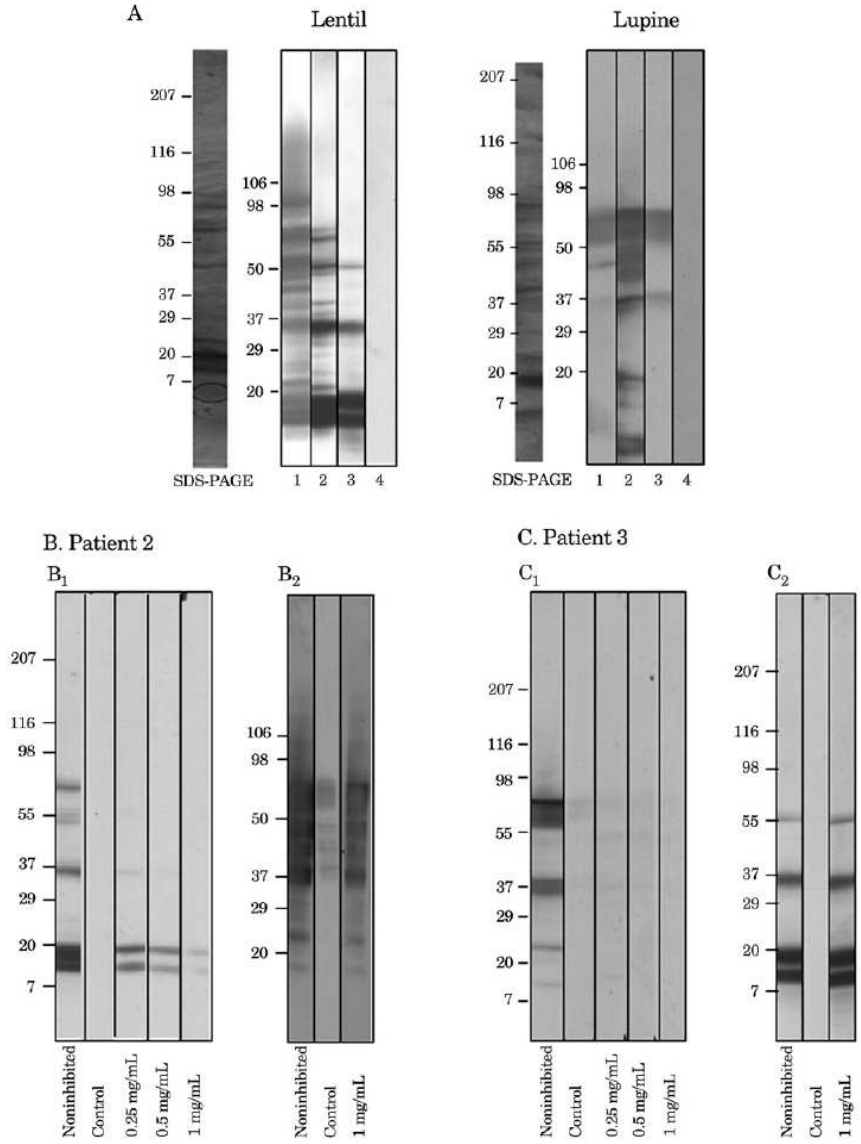

Figure 1. A, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot of lentil and lupine extracts with sera from 3 patients allergic to lentil and lupine (1-3 indicate patients; 4, negative control). B, Inhibition experiments with serum from patient 2. B_1 shows inhibition of lentil immunoblot by lupine extract (0.25-1 mg/mL; control: inhibition of lentil by lentil). B_2 shows inhibition of lupine immunoblot by lentil extract (1 mg/mL; control: inhibition of lupine by lupine). C, Inhibition experiments with serum from patient 3. C_1 shows inhibition of lupine immunoblot by lentil extract (0.25-1 mg/mL; control: inhibition of lupine by lupine). C_2 shows inhibition of lentil immunoblot by lupine extract (1 mg/mL; control: inhibition of lentil by lentil.).

sults. Lupine seems to be the primary sensitizer in patient 2, with onset of lentil allergy at 47 years of age. Given the frequent use of lupine as an ingredient in other foods,[1,4,9] he could have become sensitized through previous lupine inadvertent ingestion. In contrast, lentil seems to be the primary sensitizer in patient 3, with onset of lentil allergy at 5 years of age and a recent anaphylactic reaction. Lupine (patient 2) and lentil (patient 3) could contain most relevant IgE epitopes leading to poor inhibition by a secondary sensitizer; however, when the primary sensitizer is used as inhibitor, almost complete inhibition is achieved in a dose-dependent manner. Lentil- and lupine-allergic patients should be questioned about lupine and lentil reactivity, respectively. If there is no evidence of recent tolerance on ingestion, oral challenges should be performed.

Beatriz Cabanillas, PhD*
Jesús F. Crespo, MD, PhD*
Carmen Cuadrado, PhD†
Carmen Burbano, PhD†
Julia Rodríguez, MD, PhD*

*Servicio de Alergia
Hospital Universitario 12 de Octubre
Madrid, Spain
†Departamento de Tecnología de Alimentos
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria
Madrid, Spain
bcabanillas.hdoc@salud.madrid.org

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THE GENETIC ASSOCIATION OF THE *FPRL1* PROMOTER POLYMORPHISM WITH CHRONIC URTICARIA IN A KOREAN POPULATION

Chronic urticaria (CU) is a common immunologic disorder in the general population, but its pathogenic mechanisms are not fully understood. In general, mast cell activation has been considered to play a central role in CU.[1] A recent study[2] of urticaria suggested that neutrophil infiltration has a key role in its pathogenesis. Increased numbers of neutrophils were found in skin tissue from patients with chronic idiopathic urticaria.[3] Moreover, the role of neutrophils has been suggested for other types of urticaria, including cholinergic[4] and aspirin-intolerant urticaria.[5]

Formyl peptide receptor-like 1 (FPRL1) is a chemoattractant receptor and is mainly expressed on neutrophils, monocytes, and dendritic cells. FPRL1 plays an important role in the regulation of immune responses by the increasing migration of neutrophils and monocytes with the generation of reactive oxygen species.[6] However, there have been no published reports, to our knowledge, regarding the relation between the *FPRL1* and CU. We hypothesized that the functional variability according to *FPRL1* gene polymorphisms may be associated with CU.

To investigate the genetic association of the *FPRL1* promoter polymorphism with CU, 193 patients with CU and 231 controls were enrolled from Ajou University Hospital, Suwon, Korea; they provided informed consent, and the study protocol was approved by the institutional review board of Ajou University Hospital. All the patients and controls belonged to the same ethnic group. Patients with CU were defined as having daily itchy wheals for more than 6 weeks. Controls with no personal or family history of allergic disease were recruited. Atopy was defined as 1 or more positive skin prick test reactions to 55 common aeroallergens (Bencard Co, West Sussex, United Kingdom). The total serum IgE level was measured using the UniCAP system (Pharmacia Diagnositics, Uppsala, Sweden). Genomic DNA was extracted using the Puregene DNA Purification Kit (Gentra, Minneapolis, Minnesota). Three single nucleotide polymorphisms of the *FPRL1* gene (-6138C>T, -6136G>T, and -5840G>T) were genotyped using the SNaPshot Primer Extention kit (Applied Biosystems, Foster City, California). The sequences of the amplifying and extension primers were as follows: for *FPRL1*-61318C>T: forward, 5′-CAGGAAACAGCTATGACC-3′; reverse,

Table 1. Genotype and Haplotype Frequencies of *FPRL1* Genetic Polymorphisms[a]

	Patients With Chronic Urticaria (n=193)	Controls (n=231)	*P* value
−6138C>T			
CC	142 (73.6)	155 (67.1)	.19
CT	48 (24.9)	69 (29.9)	.46
TT	3 (1.6)	7 (3.0)	.22
−6136G>T			
GG	89 (46.1)	126 (54.5)	.02[b]
GT	73 (37.8)	89 (38.5)	.008[b]
TT	31 (16.1)	16 (6.9)	.13
−5840G>T			
GG	68 (35.2)	65 (28.1)	.21
GT	92 (47.7)	117 (50.6)	.46
TT	33 (17.1)	49 (21.2)	.20
ht1[CGT]			
ht1/ht1	11 (5.7)	18 (7.8)	.35
ht1/−	96 (49.7)	121 (52.4)	.48
−/−	86 (44.6)	92 (39.8)	.39
ht2[CGG]			
ht2/ht2	16 (8.3)	15 (6.5)	.61
ht2/−	62 (32.1)	72 (31.2)	.60
−/−	115 (59.6)	144 (62.3)	.80
ht3[CTG]			
ht3/ht3	15 (7.8)	16 (6.9)	.19
ht3/−	87 (45.1)	86 (37.2)	.11
−/−	91 (47.2)	129 (55.8)	.88
ht4[TGT]			
ht4/ht4	3 (1.6)	6 (2.6)	.02[b]
ht4/−	17 (8.8)	43 (18.6)	.01[b]
−/−	173 (89.6)	182 (78.8)	.63
ht5[TGG]			
ht5/ht5			.28
ht5/−	16 (8.3)	27 (11.7)	.28
−/−	177 (91.7)	204 (88.3)	NA

Abbreviation: NA, not applicable.
[a] Logistic regression analysis was used to analyze the single nucleotide polymorphisms and haplotypes controlling for age and sex as covariates with 3 alternative models, including codominant, dominant, and recessive model.
[b] Statistically significant.

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DISCUSIÓN

La alergia mediada por IgE es una reacción de hipersensibilidad iniciada por mecanismos inmunológicos específicos en los que están involucrados los anticuerpos IgE (Johansson et al., 2003).

Una parte importante de las investigaciones de la presente tesis doctoral está dirigida a evaluar la alergenicidad de leguminosas sometidas a tratamientos enzimáticos, térmicos y de presión. Debido a la falta de consenso sobre la metodología que se debe emplear en la evaluación de la alergenicidad de alimentos procesados, ha sido necesario desarrollar una estrategia específica basada en los métodos empleados en el análisis de la alergenicidad de hidrolizados exhaustivos de proteínas, alimentos procesados térmicamente y alimentos modificados genéticamente. La necesidad de desarrollar un modelo de evaluación del impacto del procesamiento tecnológico en la alergenicidad de los alimentos ha sido puesto de manifiesto por autores como Wichers HJ (2007) que propone un modelo basado en: 1) someter al alimento completo o a sus alergenos individuales al procesamiento tecnológico determinado y evaluar su reconocimiento por anticuerpos IgE mediante ensayos inmunoquímicos como ELISA, inmunoblot o dot-blot; 2) emplear en los experimentos sueros de un grupo suficientemente grande de pacientes con alergia clínica al alimento que se esté evaluando; 3) determinar mediante pruebas cutáneas o pruebas orales la reactividad clínica del alimento procesado en pacientes con alergia clínica al alimento en cuestión; 4) considerar incluir experimentos de digestión *in vitro*.

En investigaciones llevadas a cabo con hidrolizados enzimáticos, Restani et al (1995, 1996) realizaron experimentos de SDS-PAGE inmunoblot utilizando anticuerpos monoclonales y sueros de pacientes alérgicos a leche de vaca para detectar trazas de proteínas de leche sin hidrolizar en fórmulas lácteas sometidas a hidrólisis exhaustiva. Docena et al (2002), por su parte, evaluaron la composición alergénica de 8 hidrolizados

de leche empleando experimentos *in vitro* de ELISA, *enzyme allergosorbent test* (EAST) e inmunoblot utilizando anticuerpos monoclonales específicos y suero procedente de 15 pacientes con alergia a leche de vaca.

En cuanto al análisis de la alergenicidad de alimentos procesados térmicamente, Leduc et al (1999) detectaron a través de experimentos de SDS-PAGE inmunoblot y ELISA utilizando suero de 17 pacientes alérgicos a huevo, una reducción del reconocimiento de las proteínas de huevo por anticuerpos IgE en pasta de carne de cerdo tras un proceso de esterilización (115ºC, 90 min), mientras que el reconocimiento se mantuvo intacto en pastas de carne cruda y pasterizada (70ºC, 2 h). Wigotzki et al (2000) emplearon experimentos de inmunoblot e inhibición de EAST con sueros de pacientes con alergia clínica a avellana para demostrar que los alergenos de este fruto seco eran estables al calentamiento en horno a 100ºC pero no al calentamiento por encima de los 170ºC.

En el caso de organismos modificados genéticamente, Nordlee et al en 1996 demostraron mediante RAST e inmunoblot empleando nueve sueros de pacientes alérgicos a nuez de Brasil, así como mediante pruebas cutáneas, el potencial alergénico de una proteína codificada por un gen de nuez de Brasil transferido a soja. Los autores identifican los ensayos de inmunoblot, RAST y pruebas cutáneas como técnicas fiables para la identificación de la posible transferencia de alergenos en organismos modificados genéticamente. En otro estudio realizado por Sten et al, se emplearon métodos de inhibición de RAST, pruebas de liberación de histamina utilizando suero y sangre de pacientes sensibilizados a soja y pruebas cutáneas, para detectar diferencias en la alergenicidad potencial de 10 variedades de soja modificadas genéticamente y 8 variedades sin modificar (Sten et al., 2004). El ensayo de la seguridad de un tipo de maíz transgénico fue el objetivo de otro estudio. Los investigadores emplearon pruebas

in vitro de inmunoblot utilizando suero de 10 pacientes con alergia a ácaros que presentaban anticuerpos IgE específicos frente a Der p 7, para determinar la reactividad alergénica de un gen transferido a maíz procedente de *Bacillus thuringiensis* que presentaba cierta homología con el alergeno Der p 7 de *Dermatophagoides pteronyssinus* (Ladics et al., 2006).

Hidrólisis enzimática y su influencia en la alergenicidad de leguminosas

Las investigaciones realizadas en el presente trabajo de investigación para estudiar la influencia de la hidrólisis enzimática en la alergenicidad de leguminosas se han centrado en la utilización de dos enzimas: Alcalasa (endoproteasa) y Flavorzima (exoproteasa), las cuales se emplearon de forma individual y secuencial con extracto proteico de cacahuete y lenteja.

Hidrólisis individual con Alcalasa y Flavorzima (artículo I)

En este estudio se seleccionó la forma tostada de cacahuete para evaluar el efecto de la hidrólisis con las enzimas Alcalasa y Flavorzima empleadas individualmente, debido a que se ha demostrado ampliamente que el cacahuete tostado es más alergénico que el crudo. Maleki et al (2000b) midiendo la densidad óptica en ensayos ELISA, demostraron que el cacahuete tostado une anticuerpos IgE de pacientes alérgicos a cacahuete 90 veces más que el cacahuete crudo. Las modificaciones proteicas inducidas por la reacción de Maillard contribuirían a este aumento de la alergenicidad (Chung y Champagne, 1999; Chung y Champagne, 2001; Maleki et al., 2000b). Los resultados de nuestro trabajo mostraron que el tratamiento individual con Flavorzima durante 300

minutos da lugar a una hidrólisis del 29% y el tratamiento con Alcalasa produce una hidrólisis del 17% tras 150 minutos de reacción. En los resultados de SDS-PAGE se observó desde los primeros minutos de reacción una importante disminución de bandas proteicas en los hidrolizados con Alcalasa con respecto al extracto sin hidrolizar, detectándose la aparición de un *smear* en la zona de bajo peso molecular. Por el contrario, en los hidrolizados con Flavorzima se produjo una menor alteración del perfil electroforético de cacahuete. En los experimentos de inmunoblot y ELISA empleando un suero *pool* de 5 pacientes alérgicos a cacahuete se detectó, a los 30 minutos de hidrólisis con Alcalasa, una disminución del reconocimiento de proteínas de cacahuete por anticuerpos IgE del 98% y no se detectaron bandas en el inmunoblot. Estos resultados fueron confirmados con los experimentos de inmunoblot con sueros individuales. Por otro lado, el tratamiento individual con Flavorzima produjo un aumento del reconocimiento de las proteínas de cacahuete por parte de anticuerpos IgE a los 30 minutos de reacción detectado mediante ELISA. Sin embargo, este reconocimiento decreció al final del ensayo, tras 300 minutos de hidrólisis, dando lugar a una reducción del 65%. Estos resultados son similares a los obtenidos por Clemente et al (1999a), quienes comprobaron que el tratamiento individual con Flavorzima producía un aumento de la alergenicidad de extracto de garbanzo comparado con el extracto sin hidrolizar. Este hecho, según estos autores, podría deberse a que la hidrólisis con la endoproteasa Flavorzima produciría una mayor exposición de algunos determinantes antigénicos dando lugar a un aumento del reconocimiento por anticuerpos IgE.

Se estudió además, el efecto de Alcalasa y Flavorzima utilizadas de forma individual en el reconocimiento de alergenos específicos de cacahuete. Para ello se emplearon anticuerpos que reconocían tres de sus alergenos principales: Ara h 1, Ara h 2 y Ara h 3 (subunidad ácida y básica). Estos experimentos revelaron una disminución

en el reconocimiento de los tres alergenos desde el primer minuto de hidrólisis con Alcalasa. La hidrólisis con Flavorzima, aunque produjo una disminución del reconocimiento del alergeno Ara h 2 y la subunidad ácida de Ara h 3, afectó en menor medida al reconocimiento de los otros dos alergenos estudiados (Ara h 1 y la subunidad básica de Ara h 3), resistiendo éstos a la hidrólisis con esta enzima. La resistencia de la subunidad básica de Ara h 3 a la acción de la Flavorzima se confirmó mediante análisis de MALDI-TOF de los *spots* encontrados en los geles bidimensionales de extracto de cacahuete hidrolizado con Flavorzima. La resistencia a la hidrólisis enzimática de la subunidad básica de la globulina 11S, a la cual pertenece el alergeno Ara h 3, se ha descrito previamente en soja (Lee et al., 2007). La subunidad básica de Ara h 3 se ha identificado como un alergeno mayor de cacahuete en un grupo de niños alérgicos a este fruto seco en Italia (Restani et al., 2005).

Los resultados obtenidos en el estudio de la hidrólisis individual con Alcalasa y Flavorzima del presente trabajo de investigación permitirían afirmar que la hidrólisis con la endoproteasa Alcalasa produce un descenso del reconocimiento de las proteínas de cacahuete por anticuerpos IgE mayor que el producido por la exoproteasa Flavorzima. Así, los hidrolizados proteicos obtenidos con Alcalasa podrían constituir una alternativa a los extractos proteicos sin tratar que se emplean en la elaboración de algunos alimentos.

Hidrólisis secuencial con Alcalasa y Flavorzima (artículos I y II)

Las enzimas Alcalasa y Flavorzima se emplearon de forma secuencial para estudiar el efecto de la hidrólisis con endo-exoproteasas en la alergenicidad de lenteja y cacahuete. En lenteja, la enzima Alcalasa produjo un 24% de hidrólisis tras 3 horas de reacción, momento en el que se añadió la enzima Flavorzima, llegando a un 56% de hidrólisis al

término de la reacción (8 horas). Estos datos parecen indicar que la endoproteasa Alcalasa produjo una hidrólisis inicial que incrementó el número de secuencias diana para la exoproteasa Flavorzima. Los resultados de ELISA, empleando un *pool* de sueros de pacientes alérgicos a lenteja, mostraron que la enzima Alcalasa dio lugar a una disminución del reconocimiento de lenteja por anticuerpos IgE del 95% a los 15 segundos de reacción enzimática, momento en el que el grado de hidrólisis era del 6%. La adición de la enzima Flavorzima produjo a una reducción completa del reconocimiento IgE de lenteja. Estos resultados fueron confirmados con los experimentos de inmunoblot empleando el mismo *pool* de sueros. Sin embargo, cuando los 5 sueros de pacientes clínicamente alérgicos a lenteja se emplearon de forma individual, 4 de los mismos reconocieron alguna banda proteica en el hidrolizado de lenteja con Alcalasa durante 3 horas; sin embargo, este reconocimiento se redujo al término de la reacción con Flavorzima. Dos de los sueros reconocieron proteínas de 12, 14 y 48 kda en el hidrolizado final, lo que indica que estos alergenos presentan una mayor resistencia a la hidrólisis que otras proteínas inmunorreactivas. Estos resultados concuerdan con los obtenidos por Clemente et al (1999a), quienes obtuvieron más de un 90% de reducción de la actividad antigénica de garbanzo cuando las enzimas Alcalasa y Flavorzima se emplearon de forma secuencial. Además los autores comprobaron que la composición final de aminoácidos de los hidrolizados proteicos fue similar al material de partida (Clemente et al., 1999c). Sin embargo, la alergenicidad residual podría tener una implicación clínica importante y por tanto serán necesarios estudios *in vivo* y *ex vivo* posteriores para determinar la alergenicidad de estos hidrolizados.

También se llevó a cabo la hidrólisis secuencial de extracto proteico de cacahuete con Alcalasa y Flavorzima, pero en este caso las enzimas se utilizaron al doble de la concentración empleada en el caso de la hidrólisis de lenteja. Los resultados

de la hidrólisis mostraron que la enzima Alcalasa dio lugar a un 27% de hidrólisis tras 3 horas de reacción y la adición de la enzima Flavorzima produjo un aumento de hasta el 69% al término de la reacción (8 horas). Sin embargo, en este caso, la hidrólisis con Alcalasa a la concentración empleada (0,4 AU/g) fue suficiente para obtener una disminución del reconocimiento de las proteínas de cacahuete por anticuerpos IgE desde el primer minuto de hidrólisis tanto en inmunoblot como en ELISA. Tras 30 minutos de hidrólisis no se detectaron bandas en el inmunoblot y se consiguió una reducción completa del reconocimiento por anticuerpos IgE en ELISA. También se produjo una disminución del reconocimiento de Ara h 1, Ara h 2 y Ara h 3 desde el primer minuto de hidrólisis con Alcalasa. En este caso, la adición de la enzima Flavorzima produjo un pequeño aumento del reconocimiento por anticuerpos IgE de las proteínas de cacahuete medido en ELISA, mientras que este efecto no se observó en los experimentos de inmunoblot; por tanto, al contrario que en los resultados obtenidos en lenteja y los obtenidos por Clemente et al (1999a) en garbanzo, en el caso del extracto proteico de cacahuete, parece resultar más efectiva la hidrólisis individual con Alcalasa que la secuencial Alcalasa/Flavorzima.

Los resultados obtenidos en el estudio de la hidrólisis con Alcalasa y Flavorzima podrían abrir una vía en el desarrollo de hidrolizados de origen vegetal con propiedades alergénicas reducidas procedentes de leguminosas. Desde principios de la década de los años ochenta ha existido una gran demanda de fuentes proteicas vegetales para la elaboración de formulaciones alimentarias, sustituyendo a hidrolizados de origen animal (Clemente et al., 1999b). Los hidrolizados de leguminosas con propiedades alergénicas reducidas podrían destinarse a grupos poblacionales con necesidades específicas, tales como pacientes alérgicos a leguminosas, o bien podrían abrir el camino a la utilización de nuevas fuentes vegetales en la producción de hidrolizados.

Efecto de los tratamientos térmicos y de presión sobre la alergenicidad de alimentos

Cocción (artículos III y IV)

En el presente trabajo de investigación se estudiaron los cambios producidos en el perfil de proteínas alergénicas de lenteja y garbanzo tras tratamientos de cocción durante 15, 30 y 60 minutos. También se evaluó el efecto de la cocción durante 60 minutos en la alergenicidad de cacahuete. En el primer caso se empleó un *pool* de sueros con IgE específica a lenteja y garbanzo y sueros individuales de pacientes con IgE positiva a lenteja (25 sueros) e IgE positiva a garbanzo (24 sueros). Los resultados obtenidos en inmunoblot con dicho *pool* de sueros demostraron que los tratamientos de cocción producen pequeñas modificaciones en el perfil de proteínas alergénicas de lenteja y garbanzo comparadas con las muestras sin tratar, siendo estas modificaciones más evidentes en el tratamiento de cocción durante 60 minutos. Sin embargo, los experimentos empleando sueros individuales mostraron múltiples proteínas alergénicas en lenteja y garbanzo sometidos a cocción durante 60 minutos. La resistencia de los alergenos de lenteja a la cocción se ha puesto de manifiesto en anteriores investigaciones mediante experimentos de inhibición de ELISA e inmunoblot (Ibañez Sandin et al., 1999; Sanchez-Monge et al., 2000). También se había demostrado previamente que la composición alergénica de garbanzo crudo y cocido era similar (Martínez San Ireneo et al., 2000). Los resultados obtenidos en el presente trabajo de investigación confirman la estabilidad de la mayoría de los alergenos de lenteja y garbanzo al tratamiento de cocción.

En cacahuete, la cocción durante 60 minutos produjo pequeñas variaciones con respecto a la muestra cruda en el reconocimiento de los alergenos de cacahuete por parte

de los anticuerpos IgE de los sueros de pacientes alérgicos a cacahuete. El cambio más significativo fue la reducción en el reconocimiento de la proteína de 65 kDa (el alergeno Ara h 1) de cacahuete por parte de los anticuerpos IgE del suero *pool* de pacientes alérgicos. Estos resultados concuerdan con los obtenidos por Mondoulet et al (2005) quienes demostraron que el reconocimiento de cacahuete cocido por anticuerpos IgE fue hasta 2 veces inferior que el de cacahuete crudo o tostado. El análisis del agua de cocción demostró la existencia de proteínas alergénicas.

Tratamiento con autoclave (artículos III y IV)

Los resultados obtenidos previamente tras analizar los efectos de tratamientos como la cocción, tratamiento con autoclave, extrusión y calentamiento con microondas sobre la alergenicidad del lupino (Álvarez-Álvarez et al., 2005), nos impulsaron a plantearnos el estudio en profundidad del método más efectivo en la reducción de la alergenicidad *in vitro* de lupino: el tratamiento con autoclave.

Las investigaciones del efecto del tratamiento con autoclave del presente trabajo se han centrado en distintos parámetros de presión (1,18 y 2,56 atm), temperatura (121 y 138ºC) y tiempo (15 y 30 minutos) aplicados a cacahuete, lenteja y garbanzo. En el estudio con cacahuete, además, se profundizó en el análisis de los efectos del tratamiento con autoclave sobre alergenos específicos. Se estudiaron también los cambios en la estructura secundaria y en la digestibilidad de las proteínas de cacahuete tras el procesamiento con autoclave.

Los resultados de los experimentos *in vitro* e *in vivo* mostraron que el reconocimiento de las proteínas de cacahuete, lenteja y garbanzo por anticuerpos IgE disminuyó bajo las condiciones más extremas ensayadas en el tratamiento con autoclave (2,56 atm, 138ºC, 30 minutos) comparado con el reconocimiento IgE de cacahuete,

lenteja y garbanzo sin tratar. Sin embargo, aunque la disminución del reconocimiento IgE de proteínas de cacahuete, lenteja y garbanzo sometidas a presión a 2,56 atm, 30 minutos, fue importante, los experimentos *in vitro* mostraron que 2 de cada 10 sueros individuales de cacahuete empleados y 4 de cada 10 sueros individuales en el caso de lenteja y garbanzo reconocían alguna proteína en el extracto proteico tratado con autoclave en los experimentos de inmunoblot. Mediante ELISA, en el caso del cacahuete, se obtuvo una disminución igual o superior al 50% del reconocimiento de alergenos de cacahuete tratado con autoclave por parte de los anticuerpos IgE de 3,6 de cada 10 sueros empleados. En otras leguminosas se han descrito resultados similares; Malley et al (1975) demostraron que el tratamiento de guisantes con autoclave a 120ºC, 1 atm, durante 15 minutos producía una disminución del reconocimiento por anticuerpos IgE de sus proteínas. También, la capacidad de unir IgE de los alergenos de lupino se vio reducida por el tratamiento con autoclave a 2,56 atm durante 30 minutos, mientras que no se vio afectada por cocción, tratamiento con microondas o extrusión (Alvarez-Alvarez et al., 2005). Por el contrario, se ha demostrado que la alergenicidad de una de las proteínas de almendra (amandina) o de melocotón (Pru p 1) se mantiene tras su procesamiento con autoclave a 121ºC, 1 atm, durante 30 minutos (Brenna et al., 2000; Venkatachalam et al., 2002).

La disminución del reconocimiento de los alergenos de las leguminosas estudiadas tras el tratamiento con autoclave a 2,56 atm durante 30 minutos puede ser explicada por la pérdida de la mayoría de la estructura α-hélice que tendría lugar tras este tratamiento como demuestran los estudios de dicroísmo circular realizados en cacahuete. Se sabe que muchos de los epítopos de los alergenos mayores de cacahuete (por ejemplo: Ara h 1, Ara h 2 y Ara h 3) se encuentran localizados en las regiones con

estructura α-hélice (Barre et al., 2007; Mueller et al., 2011; Shin et al., 1998). La pérdida de esta estructura podría disminuir el reconocimiento IgE de estos alergenos.

Por otra parte, se comprobó que el cacahuete tostado procesado con autoclave, comparado con cacahuete tostado sin procesar, presentaba una mayor susceptibilidad a la digestión con tripsina, perdiendo la mayor parte de su capacidad de unir IgE del suero de los pacientes alérgicos a cacahuete. Esta mayor susceptibilidad puede ser debida a los cambios estructurales mencionados. Se ha comprobado en estudios previos que los tratamientos por calor pueden aumentar la susceptibilidad a la digestión enzimática de algunos alergenos. Morisawa et al (2009) demostró que el tratamiento de calor incrementaba la susceptibilidad del alergeno β-lactoglobulina a la digestión, facilitando así la disrupción de epítopos lineales.

Uno de los aspectos importantes que hay que tener en cuenta cuando se estudia el procesamiento térmico de alimentos es su influencia en parámetros como la solubilidad. Durante el procesamiento las proteínas pueden agregarse formando oligómeros lo cual puede producir una reducción de la solubilidad proteica (Maleki, 2004). Así, algunos tratamientos pueden alterar las proteínas alergénicas de un extracto proteico determinado debido a estos cambios en la solubilidad (Schmitt et al., 2010). De este modo no puede descartarse a priori que la disminución de la alergenicidad obtenida en los experimentos anteriormente descritos pudiera ser debida a una reducción de la solubilidad como consecuencia del procesamiento con autoclave. La fracción proteica soluble obtenida tras el tratamiento de las leguminosas y empleada en los experimentos de este trabajo podría contener una menor cantidad de proteínas alergénicas debido a una reducción de su solubilidad. Por ello, se evaluaron los perfiles de reconocimiento de los alergenos de cacahuete Ara h 1, Ara h 2 y Ara h 3 en inmunoblot empleando anticuerpos específicos, en las fracciones proteicas solubles e insolubles de cacahuete

tratado y sin tratar (Figura 2). Estos experimentos mostraron un reconocimiento mayor de Ara h 1, Ara h 2 y Ara h 3 en la fracción insoluble que en la fracción soluble. Además, se pudo observar la formación de agregados de alto peso molecular de Ara h 1 en la fracción insoluble. Estos resultados concuerdan con los obtenidos con Ara h 1 purificado en estudios previos, donde se observó que el tratamiento térmico de este alergeno da lugar a la formación de grandes complejos proteicos o agregados (Koppelman et al., 1999). Sin embargo, en el presente estudio, se observó un marcado descenso del reconocimiento de Ara h 1, Ara h 2 y Ara h 3 tras el tratamiento con autoclave más intenso ensayado (2,56 atm, 30 min) tanto en la fracción soluble como en la fracción insoluble. El análisis de las fracciones proteicas solubles e insolubles también se llevó a cabo en el caso de lenteja y garbanzo comprobándose que el extracto proteico (fracción soluble) con el que se llevaron a cabo los experimentos del estudio podía ser considerado representativo de la inmunorreactividad global de todo el alimento.

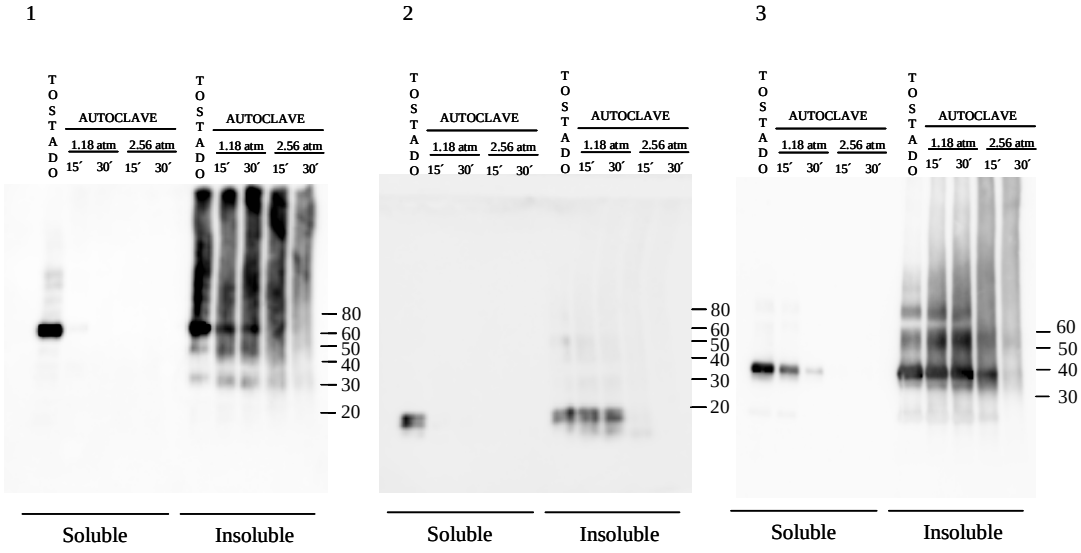

Figura 2. Inmunoblot de las fracciones solubles e insolubles de cacahuete tostado tratado y sin tratar. La detección se realizó con anticuerpos anti-Ara h 1 (1), anti-Ara h 2 (2) y anti-Ara h 3 (3).

Con los resultados obtenidos en los experimentos *in vitro* e *in vivo* expuestos se puede concluir que el tratamiento mediante autoclave a 2,56 atmósferas, 138ºC aplicado

durante 30 minutos produce una disminución del reconocimiento de los alergenos de cacahuete, garbanzo y lenteja.

Despresurización Instantánea Controlada (DIC®) (artículo V)

El presente trabajo se ha centrado también en el estudio del efecto de la tecnología DIC® en las propiedades alergénicas de leguminosas. Para ello se procesaron mediante DIC® semillas enteras de cacahuete, lenteja, garbanzo y soja, bajo condiciones de presión de 3 y 6 bares durante 1 y 3 minutos. Para comprobar los cambios en el perfil de proteínas alergénicas de las muestras tratadas y sin tratar se empleó un *pool* de sueros con IgE específica a cacahuete, lenteja, garbanzo y soja. Los resultados mostraron que los tratamientos DIC® a 3 bares durante 1 y 3 minutos y 6 bares durante 1 minuto no produjeron modificaciones relevantes en el perfil proteico y alergénico de cacahuete crudo, tostado, ni en el de soja. Sin embargo el tratamiento a 6 bares durante 3 minutos dio lugar a los cambios más relevantes en el reconocimiento de los alergenos de cacahuete crudo, tostado y soja, observándose una reducción del mismo. Estos resultados fueron más notables en cacahuete tostado y en soja que en cacahuete crudo. En el caso de lenteja y garbanzo, aunque se produjo una reducción del reconocimiento de alergenos de ambas leguminosas tras el tratamiento DIC®, las condiciones de presión y tiempo no produjeron grandes diferencias en el reconocimiento de alergenos. Este hecho resultó más patente en garbanzo que en lenteja. Las investigaciones realizadas en estudios previos con lupino demostraron que, al igual que ocurre en el presente estudio con cacahuete y soja, el procesamiento mediante DIC® a 6 bares durante 3 minutos produce una disminución del reconocimiento de los alergenos de lupino por parte del suero de pacientes con anticuerpos IgE específicos de lupino (Guillamón et al., 2008). En el presente estudio se comprobó que el tratamiento DIC® no produce una reducción

en el contenido proteico de las muestras tratadas con respecto a las muestras sin tratar, hecho que ya se había puesto de manifiesto anteriormente en otras leguminosas (Guillamón et al., 2008). Sin embargo, se observó una disminución en el contenido de proteína soluble en las muestras procesadas con DIC®. Este dato resulta importante ya que, como se ha expuesto anteriormente, ciertos tratamientos pueden alterar el perfil de proteínas alergénicas de un extracto proteico como consecuencia de los cambios en la solubilidad producidos por el tratamiento (Schmitt et al., 2010). Así, sería necesario realizar futuros experimentos dirigidos a evaluar el potencial alergénico de la fracción insoluble de las muestras tratadas con DIC®. En lupino se ha comprobado que el reconocimiento del material insoluble obtenido tras el tratamiento con DIC® por anticuerpos IgE es similar al extracto proteico soluble, mostrando una disminución en el reconocimiento de proteínas alergénicas en el tratamiento a 6 bares durante 3 minutos (Guillamón et al., 2008).

La aplicación de los resultados obtenidos en los estudios descritos requerirá la evaluación de aspectos como las características organolépticas de los alimentos procesados, así como su tolerancia en modelos murinos en primer lugar y en pacientes con alergia clínica al alimento concreto en última instancia. Recientemente se ha realizado un estudio de evaluación de las características sensoriales de productos de panadería suplementados en laboratorio con harina de lupino procesada mediante autoclave, tratamiento que produce una reducción en la alergenicidad *in vitro* de lupino (Alvarez-Alvarez et al., 2005). Los resultados indicaron que las harinas de lupino tratadas con autoclave mantenían las mismas propiedades de panificación que la harina de lupino crudo. La evaluación sensorial realizada por un panel de expertos determinó variaciones en el color de la miga del pan hecho con mezcla de harina de lupino tratada

con autoclave y evaluaron de forma muy positiva tanto su textura como su sabor (Guillamón et al., 2010b).

Tratamiento térmico en un producto dietético (artículo VI)

En el presente trabajo de investigación se analizó también la posible implicación de un tratamiento térmico en la alergenicidad de un producto dietético denominado NeOpuntia®, obtenido de hojas de chumbera (*Opuntia ficus-indica*) desecadas. Estas hojas son tradicionalmente consumidas en algunos países como por ejemplo en México, con el nombre de nopal. El producto NeOpuntia® se puede encontrar como componente de complementos dietéticos y productos adelgazantes por sus supuestos efectos beneficiosos en el metabolismo lipídico (Linarès et al., 2007). NeOpuntia® se describe en su etiqueta como un "producto no alergénico". La investigación realizada en el presente trabajo sugiere que el producto NeOpuntia® es el responsable de la grave reacción anafiláctica mediada por IgE presentada por una paciente tras el consumo de un producto adelgazante que contenía NeOpuntia® el cual es definido por el fabricante como un producto "compuesto por hojas de *Opuntia ficus-indica* procesadas mediante un procedimiento patentado libre de químicos que incluye un tratamiento de desecado por calor". Las pruebas cutáneas realizadas a la paciente, así como los experimentos de ELISA mostraron resultados positivos con NeOpuntia®, sin embargo, cuando estas mismas pruebas y experimentos se realizaron con hojas de *Opuntia ficus-indica* tanto frescas como tratadas por calor (cocción y calor seco a 90ºC y 150ºC), tratando de emular las condiciones de procesado del producto NeOpuntia®, los resultados fueron negativos.

La información existente sobre las reacciones debidas a *Opuntia ficus-indica* o sus productos procesados es escasa. Yoon et al (2004) describió un caso de dermatitis

alérgica de contacto con el fruto de *Opuntia ficus-indica* en una mujer de 43 años que se había aplicado una porción del fruto en su piel como un remedio popular. Las pruebas realizadas en la paciente con el fruto mostraron una fuerte reacción positiva. Un estudio reciente describió el caso de un paciente con una reacción anafiláctica tras el consumo de frutos de *Opuntia ficus-indica*. Las pruebas de prick-prick con la pulpa y la piel del fruto dieron lugar a resultados positivos (García-Menaya et al., 2009).

El caso descrito en el presente estudio es el primero que describe una reacción alérgica producida por la ingestión de hojas de *Opuntia ficus-indica* procesadas. Con los resultados obtenidos cabría suponer que el proceso térmico patentado para obtener el producto NeOpuntia® podría ser diferente al utilizado en el presente trabajo y podría ser el responsable de la generación de neoalergenos que habrían producido la respuesta alérgica en la paciente. El fenómeno de la generación de neoalergenos se ha descrito previamente en la literatura científica. Malanin et al (1995) describieron el caso de una paciente que tuvo una reacción anafiláctica tras el consumo de galletas que contenían nueces pacana. Las investigaciones revelaron que el suero de la paciente contenía anticuerpos IgE que reconocían específicamente alergenos presentes en nueces sometidas a tratamientos térmicos o que habían sido almacenadas y sin embargo no reconocían estos alergenos en nueces frescas. Los autores demostraron que la aparición de neoalergenos durante los tratamientos térmicos o el almacenaje de alimentos podrían ser importantes en algunas reacciones alérgicas. La formación de neoalergenos se ha comunicado también en soja por Codina et al (1998) quienes demostraron que la alergenicidad del tegumento de las semillas de esta leguminosa se ve afectada por calor, sugiriendo que las temperaturas alcanzadas durante el almacenamiento y el transporte de soja podría producir modificaciones en las proteínas y generar dos nuevos alergenos o bien incrementar la exposición de epítopos como resultado de cambios

conformacionales de las proteínas alergénicas. Rosen et al (1994) describieron el caso de un paciente sensibilizado a gamba que presentó pruebas cutáneas negativas con gamba cruda mientras que la gamba cocida produjo resultados positivos.

Estudio de la reactividad cruzada

Reactividad cruzada entre látex y maracuyá (artículo VII)

La reactividad cruzada entre látex y frutas como plátano, melón, aguacate, castaña y kiwi se ha estudiado ampliamente desde su descripción inicial como síndrome látex-frutas (Blanco et al., 1994). Sin embargo, existen otras frutas menos comunes que pueden producir una respuesta alérgica en pacientes con alergia a látex. Las quitinasas de clase I se han descrito como alergenos relevantes en el síndrome látex-frutas (Diaz-Perales et al., 1999). En el presente trabajo se describe una reacción anafiláctica experimentada por una paciente tras consumir un zumo que contenía maracuyá y que además refería síntomas cutáneos cuando usaba guantes de látex desde varios años antes. Las pruebas cutáneas realizadas con látex, el zumo que produjo la reacción y maracuyá dieron todas ellas un resultado positivo. Los niveles de IgE específica sérica fueron positivos para maracuyá (2,17 kU/L), látex (10,50 kU/L) y el alergeno heveína de látex (rHev b 6.02) (14,30 kU/L). Los resultados fueron negativos para los otros alergenos de látex ensayados (rHev b 1, 3, 5, 8, 9 y 11). A la luz de estos resultados se llevaron a cabo experimentos para determinar si existía reactividad cruzada inmunológica entre látex y maracuyá o si solo se trataba de una co-sensibilización. Los estudios de reactividad *in vitro* de inhibición de CAP entre el extracto de látex y maracuyá utilizando el suero de la paciente mostraron una inhibición completa del reconocimiento de las proteínas alergénicas de maracuyá cuando se utilizó látex como inhibidor; sin embargo, sólo se consiguió una inhibición parcial de la unión de IgE

específica de látex y heveína (rHev b 6.02) (58% y 63% respectivamente) cuando el inhibidor fue maracuyá (Figura 3). Estos resultados sugieren que todos los anticuerpos IgE específicos de maracuyá de la paciente presentan reactividad cruzada con látex pero solo algunos anticuerpos IgE específicos de látex presentes en el suero de la paciente son específicos de maracuyá. En inmunoblot los anticuerpos IgE del suero de la paciente reconocieron mayoritariamente 3 proteínas de maracuyá en torno a 55, 33 y 22 kDa, cuyo reconocimiento se inhibió por concentraciones crecientes de látex. El reconocimiento de las proteínas de 33 y 20 kDa se inhibió a concentraciones bajas de látex, mientras que el reconocimiento de la proteína de 55 kDa se inhibió a la máxima concentración de látex ensayada (Figura 3). Se ha descrito que proteínas en torno a 30 – 35 kDa de algunas frutas pertenecen a las quitinasas de clase I con un dominio N-terminal parecido a heveína implicadas en el síndrome látex-frutas (Díaz-Perales et al., 1998; Díaz-Perales et al., 1999). Díaz-Perales et al (1999) estudiaron la implicación de las quitinasas en el síndrome látex-frutas analizando el extracto proteico de 20 frutas. Los resultados en el caso del maracuyá mostraron un reconocimiento por parte de la IgE de sueros de pacientes con síndrome látex-frutas de una proteína en torno a 34 kDa que posteriormente fue reconocida también por anticuerpos anti-quitinasas. Así, en el presente estudio, la proteína de 33 kDa de maracuyá inhibida por látex podría pertenecer al grupo de las quitinasas de clase I.

Hasta la fecha existen pocos artículos donde se haya estudiado la reactividad cruzada entre látex y maracuyá. En 1992 se describió por primera vez una reacción alérgica a látex y maracuyá en un paciente, aunque no se realizaron experimentos para determinar la reactividad cruzada serológica (Ceuppens et al., 1992). La primera evidencia de la existencia de anticuerpos IgE que reconocían epítopos de látex y de maracuyá la encontramos en el estudio de Brehler et al (1997) quienes por medio de

experimentos de inhibición de RAST utilizando el suero de un paciente con alergia a látex y sensibilización inmunológica a maracuyá, observaron una inhibición completa de extracto de maracuyá utilizando extracto de látex como inhibidor y una inhibición débil de látex cuando el inhibidor ensayado fue extracto de maracuyá.

El estudio del presente trabajo es el primero en demostrar las implicaciones clínicas de la reactividad inmunológica entre látex y maracuyá. Los resultados obtenidos muestran la importancia de evaluar el riesgo de reacción a maracuyá en pacientes alérgicos a látex; la ingestión de frutas exóticas, como componentes de zumos por ejemplo, parece ser cada vez más frecuente y puede constituir un riesgo de reacciones alérgicas en algunos pacientes con alergia a látex.

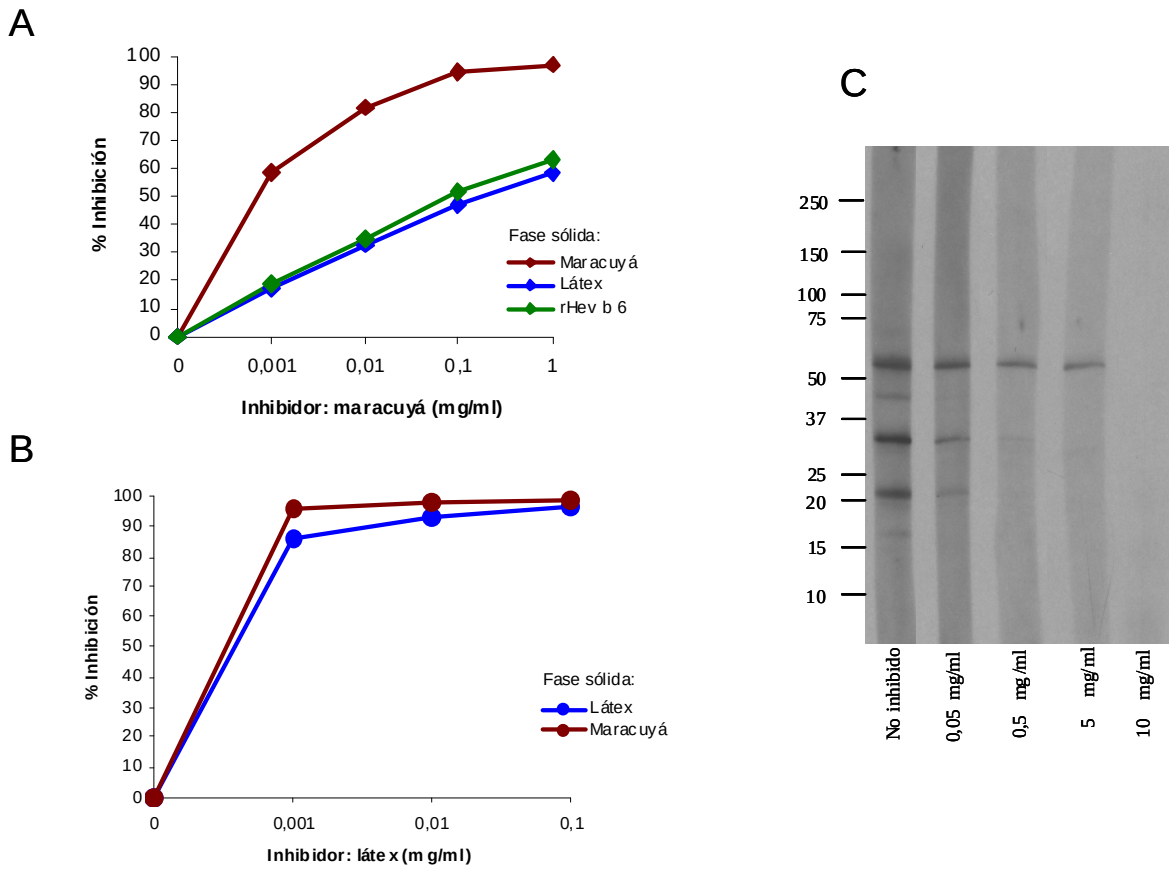

Figura 3. Inhibición de CAP de maracuyá, látex y rHev b 6 utilizando maracuyá como inhibidor (A) e inhibición de CAP de látex y maracuyá empleando látex como inhibidor (B). En la figura C se muestra el inmunoblot inhibición de maracuyá inhibido por concentraciones crecientes de látex. En todos los ensayos el suero de la paciente alérgica a látex y maracuyá se empleó a una dilución final de 1:5.

Reactividad cruzada entre lupino y lenteja (artículo VIII)

En el presente trabajo se analizó la reactividad cruzada entre dos leguminosas: lenteja y lupino. La alergia a lupino ha ido aumentando a medida que se ha incrementado el uso de esta leguminosa en productos alimentarios. La harina de lupino se ha introducido como suplemento de harina de trigo especialmente en pan y pasta debido a sus propiedades nutricionales (Guillamón et al., 2008; Marchesi et al., 2008). Una parte importante de los casos publicados de alergia a lupino se han descrito en pacientes alérgicos a cacahuete (Faeste et al., 2004; Hefle et al., 1994 Moneret-Vautrin et al., 1999) demostrándose la existencia de reactividad cruzada serológica. Sin embargo, muy pocos trabajos han estudiado la asociación de la alergia a lupino con otras leguminosas (Matheu et al., 1999). El presente trabajo de investigación describe el caso de una paciente (nº 1) alérgica a lenteja y cacahuete que tuvo una reacción anafiláctica tras el consumo de semillas de lupino. Tanto las pruebas cutáneas con lupino como la IgE específica a esta leguminosa dieron resultados positivos en la paciente. Esta reacción nos llevó a evaluar la posible reactividad cruzada entre lupino y lenteja en 5 pacientes consecutivos con alergia clínica a lenteja (confirmada por una provocación oral doble ciego controlada con placebo positiva o bien una reacción anafiláctica grave) y que no habían consumido lupino con anterioridad. De los 5 pacientes evaluados, dos de ellos (nº 2 y 3) presentaron pruebas inmunológicas (pruebas cutáneas e IgE específica en sangre) y provocación oral doble ciego controlada con placebo con resultado positivo con harina de lupino. Ninguno de estos dos pacientes era alérgico a cacahuete. El paciente número 4 presentó reactividad inmunológica positiva a lupino (pruebas cutáneas e IgE específica positivas) pero una provocación oral negativa con lupino. Los pacientes 5 y 6 presentaron reactividad inmunológica y clínica negativa a lupino. En la investigación realizada mediante inmunoblot inhibición para determinar si las

reacciones a lenteja y lupino de los pacientes 2 y 3 se debían a la reactividad cruzada entre estas dos leguminosas o bien se trataba de una co-sensibilización, se constató la existencia de reactividad cruzada entre ambas leguminosas. Sin embargo, las características de esta reactividad fueron muy distintas en cada uno de los dos pacientes. Cuando se empleó el suero del paciente 2, se observó una inhibición casi completa del reconocimiento de alergenos de lenteja utilizando lupino como inhibidor. Sin embargo no se produjo una inhibición del reconocimiento de lupino cuando la lenteja fue utilizada como inhibidor. El suero de la paciente 3 mostró resultados opuestos: se produjo una inhibición completa del reconocimiento alergenos de lupino cuando se empleó lenteja como inhibidor y no se consiguió tal inhibición en lenteja cuando el inhibidor fue lupino (Figura 4). Estos resultados parecen indicar que el lupino sería el sensibilizante primario en el paciente 2, y el sensibilizante secundario la lenteja, con una edad de comienzo de la alergia a lenteja en el paciente de 47 años (tenía 49 años en el momento del estudio). Debido al frecuente uso de lupino como aditivo en numerosos alimentos y que en ocasiones se puede encontrar como alergeno oculto (Rojas-Hijazo et al., 2006) podría haber existido una sensibilización del paciente a través de la ingesta inadvertida de lupino. En contraposición, la lenteja parece ser el sensibilizante primario en la paciente 3, con un comienzo de la alergia a lenteja a los 5 años de edad (tenía 27 años en el momento del estudio). El lupino, en el paciente 2 y la lenteja en la paciente 3, podría contener la mayor parte de los epítopos reconocidos por anticuerpos IgE, produciendo una inhibición pobre por parte del sensibilizante secundario; sin embargo, cuando el sensibilizante primario se emplea como inhibidor, se produce una completa inhibición del secundario. Este fenómeno se ha descrito previamente en polen y en frutas. Kazemi-Shirazi et al (2000) estudiaron a pacientes con síndrome alérgico oral y comprobaron que los alergenos de polen de abedul y *Phleum pratense* inhibían el

reconocimiento de anticuerpos IgE de alergenos de manzana, zanahoria, apio, avellana, kiwi y melocotón, mientras que los alergenos de estos alimentos inhibieron pobremente el reconocimiento de los alergenos de polen. De este modo se evidenció el papel del polen como sensibilizante primario en pacientes con síndrome de alergia oral a frutas.

Los resultados obtenidos en el presente trabajo muestran reactividad inmunológica a lupino en 4 de 6 pacientes alérgicos a lenteja y reactividad clínica en 3 de 6. Estos hallazgos ponen de manifiesto la necesidad de estudiar la posible presencia de alergia a lupino en pacientes con alergia clínica a lenteja y viceversa.

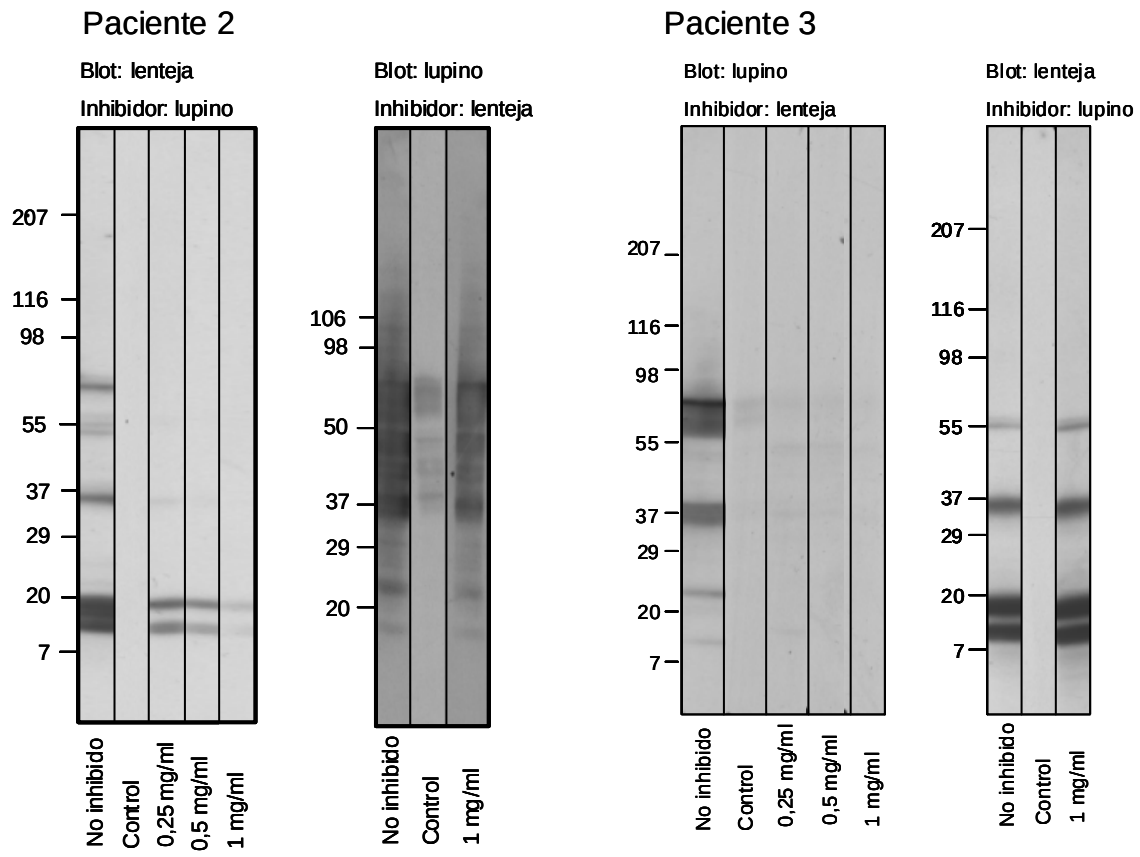

Figura 4. Inmunoblots inhibición lenteja-lupino llevados a cabo con sueros de los pacientes 2 y 3.

CONCLUSIONES

1. La hidrólisis con la enzima endoproteasa Alcalasa produce un descenso del reconocimiento de los alergenos de cacahuete por anticuerpos IgE mayor que el producido por la hidrólisis con la enzima exoproteasa Flavorzima. La hidrólisis secuencial endo-exoproteasa resulta efectiva para la reducción del reconocimiento de alergenos de lenteja por anticuerpos IgE.

2. Se ha confirmado que los alergenos de lenteja, garbanzo y cacahuete son en su mayoría estables a los tratamientos de cocción.

3. El tratamiento con autoclave a 2,56 atmósferas durante 30 minutos produce una disminución del reconocimiento de proteínas alergénicas de cacahuete, lenteja y garbanzo por los anticuerpos IgE del 78%, 56% y 59% de los sueros individuales empleados en cacahuete, lenteja y garbanzo respectivamenete.

4. Los estudios de dicroismo circular realizados en cacahuete sugieren que el tratamiento mediante autoclave produce la pérdida de la mayoría de las estructuras α-hélice, lo que podría explicar el descenso del reconocimiento de proteínas alergénicas de cacahuete tras el tratamiento.

5. El tratamiento con autoclave incrementa la susceptibilidad del cacahuete a la digestión con tripsina, perdiendo la mayor parte de su capacidad de unir IgE del suero de los pacientes alérgicos a cacahuete.

6. El reconocimiento por anticuerpos específicos de los alergenos mayores de cacahuete Ara h 1, Ara h 2 y Ara h 3 tras el tratamiento de autoclave a 2,56 atmósferas durante 30 minutos disminuye tanto en la fracción proteica soluble como en la insoluble.

7. El tratamiento de Despresurización Instantánea Controlada (DIC) a 3 bares durante 1 y 3 minutos y 6 bares durante 1 minuto no produce modificaciones relevantes en el perfil proteico y alergénico de cacahuete y soja. Sin embargo el tratamiento a 6 bares durante 3 minutos produce una disminución del reconocimiento de los alergenos de estas leguminosas por anticuerpos IgE. La tecnología DIC es menos eficaz en garbanzo y lenteja.

8. El procesamiento térmico de las hojas de *Opuntia ficus-indica* podría dar lugar a la generación de neoalergenos, posible responsable de reacciones alérgicas por la ingestión de dicho alimento como producto dietético.

9. Se ha realizado un estudio de la reactividad cruzada a nivel de anticuerpos IgE entre lupino y lenteja y entre látex y maracuyá, y se ha demostrado la implicación clínica de dicha reactividad cruzada.

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